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The attached documents are exact copies of the international patent application described on the following page, as originally filed.

Les documents fixés à cette attestation sont conformes à la version initialement déposée de la demande de brevet international spécifiée à la page suivante.

Den Haag, den
The Hague,
La Haye, le 04 12 2003

Der Präsident des Europäischen Patentamts
Im Auftrag
For the President of the European Patent Office
Le Président de l'Office européen des brevets
p.o.

M. Claudepierre

Patentanmeldung Nr.
Patent application no.
Demande de brevet n° PCT/EP 03/07313

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Sheet 2 of the certificate
Page 2 de l'attestation

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Titre de l'invention: STABILIZED SINGLE DOMAIN ANTIBODIES

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PCT REQUEST

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III-2	**Applicant and/or inventor**	
III-2-1	This person is:	applicant and inventor
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V	**Designation of States**	
V-1	Regional Patent (other kinds of protection or treatment, if any, are specified between parentheses after the designation(s) concerned)	AP: GH GM KE LS MW MZ SD SL SZ TZ UG ZM ZW and any other State which is a Contracting State of the Harare Protocol and of the PCT EA: AM AZ BY KG KZ MD RU TJ TM and any other State which is a Contracting State of the Eurasian Patent Convention and of the PCT EP: AT BE BG CH&LI CY CZ DE DK EE ES FI FR GB GR HU IE IT LU MC NL PT SE SI SK TR and any other State which is a Contracting State of the European Patent Convention and of the PCT OA: BF BJ CF CG CI CM GA GN GQ GW ML MR NE SN TD TG and any other State which is a member State of OAPI and a Contracting State of the PCT

PCT REQUEST ABL-013-PCT

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V-2	National Patent (other kinds of protection or treatment, if any, are specified between parentheses after the designation(s) concerned)	AE AG AL AM AT AU AZ BA BB BG BR BY BZ CA CH&LI CN CO CR CU CZ DE DK DM DZ EC EE ES FI GB GD GE GH GM HR HU ID IL IN IS JP KE KG KP KR KZ LC LK LR LS LT LU LV MA MD MG MK MN MW MX MZ NI NO NZ OM PH PL PT RO RU SC SD SE SG SK SL TJ TM TN TR TT TZ UA UG US UZ VC VN YU ZA ZM ZW	
V-5	**Precautionary Designation Statement** In addition to the designations made under items V-1, V-2 and V-3, the applicant also makes under Rule 4.9(b) all designations which would be permitted under the PCT except any designation(s) of the State(s) indicated under item V-6 below. The applicant declares that those additional designations are subject to confirmation and that any designation which is not confirmed before the expiration of 15 months from the priority date is to be regarded as withdrawn by the applicant at the expiration of that time limit.		
V-6	**Exclusion(s) from precautionary designations**	NONE	
VI	**Priority claim**	NONE	
VII-1	**International Searching Authority Chosen**	European Patent Office (EPO) (ISA/EP)	
VIII	**Declarations**	Number of declarations	
VIII-1	Declaration as to the identity of the inventor	-	
VIII-2	Declaration as to the applicant's entitlement, as at the international filing date, to apply for and be granted a patent	-	
VIII-3	Declaration as to the applicant's entitlement, as at the international filing date, to claim the priority of the earlier application	-	
VIII-4	Declaration of inventorship (only for the purposes of the designation of the United States of America)	-	
VIII-5	Declaration as to non-prejudicial disclosures or exceptions to lack of novelty	-	
IX	**Check list**	number of sheets	electronic file(s) attached
IX-1	Request (including declaration sheets)	4	-
IX-2	Description	59	-
IX-3	Claims	8	-
IX-4	Abstract	1	EZABST00.TXT
IX-5	Drawings	20	-
IX-7	TOTAL	92	
	Accompanying Items	paper document(s) attached	electronic file(s) attached
IX-8	Fee calculation sheet	✓	-
IX-17	PCT-EASY diskette	-	Diskette

STABILIZED SINGLE DOMAIN ANTIBODIES

FIELD OF THE INVENTION

The present invention provides single domain antibodies, comprising heavy chain antibodies, having improved stability *in vivo* and their use in diagnosis and therapy.

BACKGROUND OF THE INVENTION

Polypeptide therapeutics and in particular antibody-based therapeutics have significant potential as drugs because they have exquisite specificity to their target and a low inherent toxicity. However, in order to be effective as therapeutic agent, their pharmacokinetic profile should be optimized. The majority of current antibody applications are for acute disorders. There are however significant opportunities to develop antibody therapeutics for chronic conditions. This will require large doses of protein over a long period of time. Since the cost of antibody production in mammalian cells is high, the development of traditional antibody therapeutics for these applications has been discouraged. An alternative approach has been to express fragments of antibodies such as Fab's or single-chain Fv's in microbial expression systems such as yeast and bacteria. These fragments however have very short circulation times *in vivo*.

Some of the initial approaches to increase the circulation in the bloodstream of proteins and peptides were based on chemical modification, such as pegylation (US 4,179,337). Examples of such products are PEG-Intron, i.e. pegylated interferon alpha-2b for the treatment of HCV, and treatment of chronic disorder with PEG-modified antibodies (A.P Chapman, Adv.Drug Delivery Reviews (2002), 54, 531-545). Such chemical methods, however, suffer from a number of disadvantages, such as inactivation of the target protein or peptide due to the chemical modification of certain amino acid side chains, instability of the target protein/peptide during the chemical reaction.

To overcome these limitations, alternative approaches have been developed, first of all by using non-conventional or modified proteins, secondly by using alternative methods to increase halflife *in vivo*. Stabilisation of the protein drug can therefore be carried out by choosing an inherently stable protein scaffold and providing methods to bind such scaffold

to plasma proteins which occur in high concentrations, such as immunoglobulins or albumin. Binding to plasma protein can be an effective means to improving the pharmacokinetic properties of molecules in general. More precisely, binding to albumin to improve the t1/2 of proteins has been described: M.S. Dennis *et al.* (*J. Biol. Chem.* 33, 2383-90, 2002) isolated peptides that specifically bind serum albumin. When bound to a Fab molecule, half-lives comparable to pegylated Fab's were obtained. Peptide ligands having affinity for IgG or serum albumin have been disclosed (WO 01/45746), as well as a process to extending the halve-life *in vivo* of proteins or peptides by covalent coupling to polypeptides capable of binding to a serum protein (WO 91/01743). The drawback of such approach is that the peptides have to fold properly and be accessible to binding to serum albumin when fused to the therapeutic protein. Therefore, these peptides are inherently unstable and have affinities in the submicromolar range rather than subnanomolar or low nanomolar range, as is the case with conventional antibodies. As part of a larger protein, such as an conventional antibody molecule, binding of these peptides to albumin may be sterically hindered.

An alternative hybrid molecule with two functional units is based on an heterospecific antibody. Such a hybrid would consist of a bifunctional or heterospecific antibody construct with one entity having specificity and affinity for the target, the second entity having specificity and affinity for a serum protein, such as albumin. However, such heterospecific constructs based on conventional antibodies or Fab fragments have several important drawbacks: these are complex, large molecules composed of two polypeptide chains (VH and VL) and therefore difficult and expensive to produce in high amounts in mammalian expression systems. Furthermore, producing bifunctional antibodies composed of 4 chains (2 VH's and 2 VL's) have the inherent risk of resulting in molecules with the unproductive VH-VL combinations and consequently loss of activity. Several alternatives have been tried with mixed results based on peptide derivatives of conventional antibodies, such as diabodies and bifunctional scFv's (WO0220615; WO9413804; WO9119739; WO9409131)).

A complex of interferon with a monoclonal antibody to increase the serum half-life of interferon has been described in US 5,055,289. Such approach has the inherent risk of impairing the biological activity of the interferon since the size of the construct raises the problem of steric hindrance.

THE AIMS OF THE PRESENT INVENTION

It is an aim of the present invention to provide therapeutic heterospecific antibody polypeptide constructs which overcome the problems of therapeutic antibodies of the art namely, low half-life *in vivo*, poor folding, low expression, and poor stability. It is a further aim of the present invention to provide methods for providing said heterospecific antibodies .

SUMMARY OF THE INVENTION

One embodiment of the present invention is a polypeptide construct comprising:

- one or more single domain antibodies each directed against one or more therapeutic and/or diagnostic targets, and
- one or more single domain antibodies each directed against one or more serum proteins.

Another embodiment of the present invention is a polypeptide construct as defined above directed against a single target wherein said anti-target single domain antibodies do not share the same sequence.

Another embodiment of the present invention is a polypeptide construct as defined above directed against a single serum protein wherein said anti-serum protein single domain antibodies do not share the same sequence.

Another embodiment of the present invention is a polypeptide construct as defined above wherein said single domain antibodies are *Camelidae* VHHs antibodies.

Another embodiment of the present invention is a polypeptide construct as defined above wherein said one or more serum protein are any of serum albumin, serum immunoglobulins, thyroxine-binding protein, transferring, or fibrinogen or a fragment thereof.

Another embodiment of the present invention is a polypeptide as defined above wherein said one or more single domain anti-serum protein antibodies correspond to one or more sequences represented by any of SEQ ID NOs: 1 to 3.

Another embodiment of the present invention is a polypeptide construct as defined above directed against a single target, wherein said target is Tumour Necrosis Factor-alpha.

Another embodiment of the present invention is a polypeptide construct as defined above wherein said one or more single domain anti-target antibodies correspond to one or more sequences represented by any of SEQ ID NOs: 17 to 29.

Another embodiment of the present invention is a polypeptide construct as defined above corresponding to the sequence represented by any of SEQ ID NO: 4 to 6.

Another embodiment of the present invention is a polypeptide construct as defined above, wherein said polypeptide construct is a homologous sequence of said polypeptide construct, a functional portion thereof, of an homologous sequence of a functional portion thereof.

Another embodiment of the present invention is a nucleic acid encoding a polypeptide construct as defined above.

Another embodiment of the present invention is a polypeptide construct as defined above, or a nucleic acid as defined above for use in the treatment, prevention and/or alleviation of disorders relating to inflammatory processes.

Another embodiment of the present invention is a use of a polypeptide construct as defined above, or a nucleic acid as defined above for the preparation of a medicament for the treatment, prevention and/or alleviation of disorders relating to inflammatory processes.

Another embodiment of the present invention is a polypeptide construct or nucleic acid as defined above or a use of a polypeptide construct as defined above wherein said disorders are any of rheumatoid arthritis, Crohn's disease, ulcerative colitis and multiple sclerosis.

Another embodiment of the present invention is a polypeptide construct or nucleic acid as defined above or a use of a polypeptide construct as defined above wherein said polypeptide construct is administered intravenously, orally, sublingually, topically, nasally, vaginally, rectally or by inhalation.

Another embodiment of the present invention is a polypeptide construct as defined above directed against a single target wherein said target is vWF

Another embodiment of the present invention is a polypeptide construct as defined above directed against a single target wherein said target is collagen.

Another embodiment of the present invention is a polypeptide construct as defined above wherein said one or more anti-target single domain antibodies correspond to one or more sequences represented by any of SEQ ID NOs: 30 to 39.

Another embodiment of the present invention is a polypeptide construct as defined above corresponding to the sequence represented by any of SEQ ID NOs: 40 to 42.

Another embodiment of the present invention is a polypeptide construct as defined above, wherein said polypeptide construct is a homologous sequence of said polypeptide construct, a functional portion thereof, of an homologous sequence of a functional portion thereof.

Another embodiment of the present invention is a nucleic acid encoding a polypeptide construct as defined above.

Another embodiment of the present invention is a polypeptide construct as defined above, or a nucleic acid as defined above for use in the treatment, prevention and/or alleviation of disorders or conditions relating to platelet-mediated aggregation or dysfunction thereof.

Another embodiment of the present invention is a use of a polypeptide construct as defined above, or a nucleic acid as defined above for the preparation of a medicament for the treatment, prevention and/or alleviation of disorders or conditions relating to platelet-mediated aggregation or dysfunction thereof.

Another embodiment of the present invention is a polypeptide construct or nucleic acid as defined above or a use of a polypeptide construct or nucleic acid as defined above wherein said disorders are any of cerebral ischemic attack, unstable angina pectoris, cerebral

infarction, myocardial infarction, peripheral arterial occlusive disease, restenosis, and said conditions are those arising from coronary by-pass graft, or coronary artery valve replacement and coronary interventions such angioplasty, stenting, or atherectomy.

Another embodiment of the present invention is a polypeptide construct or nucleic acid as defined above or a use of a polypeptide construct as defined above wherein said polypeptide construct is administered intravenously, orally, sublingually, topically, nasally, vaginally, rectally or by inhalation.

Another embodiment of the present invention is a polypeptide construct as defined above directed against a single target wherein said target is IgE.

Another embodiment of the present invention is a polypeptide construct as defined above wherein said one or more anti-target single domain antibodies correspond to one or more sequences represented by any of SEQ ID NOs: 7 to 16.

Another embodiment of the present invention is a polypeptide construct as defined above corresponding to the sequence represented by any of SEQ ID NOs: 43 to 45.

Another embodiment of the present invention is a polypeptide construct as defined above, wherein said polypeptide construct is a homologous sequence of said polypeptide construct, a functional portion thereof, of an homologous sequence of a functional portion thereof.

Another embodiment of the present invention is a nucleic acid encoding a polypeptide construct as defined above.

Another embodiment of the present invention is a polypeptide construct as defined above, or a nucleic acid as defined above for use in the treatment, prevention and/or alleviation of disorders or conditions relating to allergic reactions.

Another embodiment of the present invention is a use of a polypeptide construct as defined above, or a nucleic acid as defined above for the preparation of a medicament for the

treatment, prevention and/or alleviation of disorders or conditions relating to allergic reactions.

Another embodiment of the present invention is a polypeptide construct or nucleic acid as defined above or a use of a polypeptide construct or nucleic acid as defined above wherein said disorders are any of hay fever, asthma, atopic dermatitis, allergic skin reactions, allergic eye reactions and food allergies.

Another embodiment of the present invention is a polypeptide construct or nucleic acid as defined above or a use of a polypeptide construct as defined above wherein said polypeptide construct is administered intravenously, orally, sublingually, topically, nasally, vaginally, rectally or by inhalation.

Another embodiment of the present invention is a polypeptide construct as defined above directed against a single target wherein said target is IFN-gamma .

Another embodiment of the present invention is a polypeptide construct as defined above wherein said anti-target single domain antibodies are anti-IFN-gamma VHHs.

Another embodiment of the present invention is a polypeptide construct as defined above wherein said anti-target single domain antibodies and said anti-serum protein antibodies are both VHHs .

Another embodiment of the present invention is a polypeptide construct as defined above, wherein said polypeptide construct is a homologous sequence of said polypeptide construct, a functional portion thereof, of an homologous sequence of a functional portion thereof.

Another embodiment of the present invention is a nucleic acid encoding a polypeptide construct as defined above.

Another embodiment of the present invention is a polypeptide construct as defined above, or a nucleic acid as defined above for use in the treatment, prevention and/or alleviation of disorders or conditions wherein the immune system is over-active.

Another embodiment of the present invention is a use of a polypeptide construct as defined above, or a nucleic acid as defined above for the preparation of a medicament for the treatment, prevention and/or alleviation of disorders or conditions wherein the immune system is over-active.

Another embodiment of the present invention is a polypeptide construct or nucleic acid as defined above or a use of a polypeptide construct or nucleic acid as defined above wherein said disorders are any of Crohn's disease, autoimmune disorders and organ plant rejection in addition inflammatory disorders such as rheumatoid arthritis, Crohn's disease, ulcerative colitis and multiple sclerosis.

Another embodiment of the present invention is a polypeptide construct or nucleic acid as defined above or a use of a polypeptide construct as defined above wherein said polypeptide construct is administered intravenously, orally, sublingually, topically, nasally, vaginally, rectally or by inhalation.

Another embodiment of the present invention is a composition comprising a polypeptide construct as defined above, or a nucleic acid encoding said polypeptide construct and a pharmaceutically acceptable vehicle.

Another embodiment of the present invention is a composition comprising a polypeptide construct as defined above, or a nucleic acid encoding said polypeptide construct and a pharmaceutically acceptable vehicle.

Another embodiment of the present invention is a composition comprising a polypeptide construct as defined above, or a nucleic acid encoding said polypeptide construct and a pharmaceutically acceptable vehicle.

Another embodiment of the present invention is a polypeptide construct as defined above directed against a single target wherein said target is involved in a disease process.

Another embodiment of the present invention is a polypeptide construct as defined above, wherein said polypeptide construct is a homologous sequence of said polypeptide construct, a functional portion thereof, of an homologous sequence of a functional portion thereof.

Another embodiment of the present invention is a nucleic acid encoding a polypeptide construct as defined above.

Another embodiment of the present invention is a polypeptide construct as defined above, or a nucleic acid as defined above for use in the treatment, prevention and/or alleviation of disorders or conditions in which the target is involved.

Another embodiment of the present invention is a use of a polypeptide construct as defined above, or a nucleic acid as defined above for the preparation of a medicament for the treatment, prevention and/or alleviation of disorders or conditions in which the target is involved.

Another embodiment of the present invention is a polypeptide construct as defined above, or a nucleic acid as defined above for use in treating, preventing and/or alleviating the symptoms of a disease requiring a therapeutic or diagnostic compound which is not rapidly cleared from the circulation.

Another embodiment of the present invention is a use of a polypeptide construct as defined above, or a nucleic acid as defined above for the preparation of a medicament for treating, preventing and/or alleviating the symptoms of a disease requiring a therapeutic or diagnostic compound which is not rapidly cleared from the circulation.

Another embodiment of the present invention is a polypeptide construct as defined above, or a nucleic acid as defined above for use in treating, preventing and/or alleviating the symptoms of a disease requiring a therapeutic or diagnostic compound which remains active in the circulation for extended periods of time.

Another embodiment of the present invention is a use of a polypeptide construct as defined above, or a nucleic acid as defined above for the preparation of a medicament for treating,

preventing and/or alleviating the symptoms of a disease requiring a therapeutic or diagnostic compound which is remains active in the circulation for extended periods of time.

Another embodiment of the present invention is a polypeptide construct or nucleic acid as defined above, or use of a polypeptide construct or nucleic acid as defined above, wherein said polypeptide construct is administered intravenously, orally, sublingually, topically, nasally, vaginally, rectally or by inhalation.

Another embodiment of the present invention is a composition comprising a polypeptide construct as defined above, or a nucleic acid as defined above and a pharmaceutically acceptable vehicle.

Another embodiment of the present invention is a method of producing a polypeptide as defined above comprising

(a) culturing host cells comprising nucleic acid capable of encoding a polypeptide as defined above, under conditions allowing the expression of the polypeptide, and,
(b) recovering the produced polypeptide from the culture.

Another embodiment of the present invention is a method as defined above, wherein said host cells are bacterial or yeast.

Another embodiment of the present invention is a method for prolonging the half-life of a single domain antibody in the blood steam of a subject, said antibody directed against a therapeutic and/or diagnostic target by joining thereto one or more single domain antibodies each directed against a serum protein.

Another embodiment of the present invention is a method as defined above wherein said anti-target single domain antibodies do not share the same sequence.

Another embodiment of the present invention is a method as defined above wherein said anti-serum protein single domain antibodies do not share the same sequence.

Another embodiment of the present invention is a method as defined above wherein said single domain antibodies are *Camelidae* VHHs antibodies.

Another embodiment of the present invention is a method as defined above wherein said serum protein is any of serum albumin, serum immunoglobulins, thyroxine-binding protein, transferring, or fibrinogen or a fragment thereof.

Another embodiment of the present invention is a method as defined above wherein said serum protein comprises a sequence corresponding to any of SEQ ID NOs: 1 to 3, a homologous sequence, a functional portion thereof, or a homologous sequence of a functional portion thereof.

Another embodiment of the present invention is a composition comprising a polypeptide as defined above or a nucleic acid capable of encoding said polypeptide and a pharmaceutically acceptable vehicle.

DETAILED DESCRIPTION OF THE INVENTION

The present invention relates to a polypeptide construct comprising one or more single domain antibodies each directed against a serum protein(s) of a subject, and one or more single domain antibodies each directed against a target molecule(s) and the finding that the construct has a significantly prolonged half-life in the circulation of said subject compared with the half-life of the anti-target single domain antibody when not part of a construct. Single domain antibodies may be any of the art, or any future single domain antibodies. Examples include, but are not limited to, heavy chain antibodies, antibodies naturally devoid of light chains, single domain antibodies derived from conventional 4-chain antibodies, engineered antibodies and single domain scaffolds other than those derived form antibodies. According to one aspect of the invention, a single domain antibodies as used herein is a VHH molecule - single domain heavy chain antibodies derived from antibodies raised in *Camelidae* species, for example in camel, dromedary, alpaca and guanaco.

The one or more single domain antibodies of the polypeptide construct which are directed against a target may be of the same sequence. Alternatively they may not all have the same sequence. It is within the scope of the invention that a polypeptide construct comprises anti-target single domain antibodies which do not all share the same sequence, but which are directed against the same target, or fragment thereof, one or more antigens thereof.

The one or more single domain antibodies of the polypeptide construct which are directed against a serum protein may be of the same sequence. Alternatively they may not all have the same sequence. It is within the scope of the invention that a polypeptide construct comprises anti-serum protein single domain antibodies which do not all share the same sequence, but which are directed against the same target, or fragment thereof, one or more antigens thereof.

In another embodiment, one or more anti-target single domain antibodies of the polypeptide construct may be directed to more than one target (*e.g.* vWF and collagen). Similarly, the anti-serum protein single domain antibodies of the polypeptide construct may be directed against more than one serum protein (*e.g.* serum albumin and fibrinogen).

VHHs, according to the present invention, and as known to the skilled addressee are heavy chain variable domains derived from immunoglobulins naturally devoid of light chains such as those derived from *Camelids* as described in WO9404678 (and referred to hereinafter as VHH domains). VHH molecules are about 10x smaller than IgG molecules. They are single polypeptides and very stable, resisting extreme pH and temperature conditions. Moreover, they are resistant to the action of proteases which is not the case for conventional antibodies. Furthermore, *in vitro* expression of VHHs produces high yield, properly folded functional VHHs. In addition, antibodies generated in *Camelids* will recognize epitopes other than those recognised by antibodies generated *in vitro* through the use of antibody libraries or via immunisation of mammals other than *Camelids* (WO 9749805). As such, anti-albumin VHH's may interact in a more efficient way with serum albumin which is known to be a carrier protein. As a carrier protein some of the epitopes of serum albumin may be inaccessible by bound proteins, peptides and small chemical compounds. Since VHH's are known to bind into 'unusual' or non-conventional epitopes such as cavities (WO9749805), the affinity of such VHH's to circulating albumin may be increased.

The present invention also relates to the finding that a polypeptide construct comprising one or more VHHs directed against one or more serum proteins of a subject, and one or more VHHs directed against one or more target molecule of said subject surprisingly has significantly prolonged half-life in the circulation of said subject compared with the half-life of the anti-target VHH when not part of said construct. Furthermore, the said construct was found to exhibit the same favourable properties of VHHs such as high stability, extreme pH resistance, high temperature stability and high target affinity.

A target according to the invention is any biological substance capable of binding to a polypeptide construct of the invention. Targets may be, for example, proteins, peptides, nucleic acids, oligonucleic acids, saccharides, polysaccharides, glycoproteins. Examples include, but are not limited to therapeutic targets, diagnostic targets, receptors, receptor ligands, viral coat proteins, immune system proteins, hormones, enzymes, antigens, cell signaling proteins, or a fragment thereof. Targets may be native protein or a fragment thereof, a homologous sequence thereof, a functional portion thereof, or a functional portion of an homologous sequence.

The present invention also relates to a polypeptide construct comprising one or more VHHs each directed against one or more serum proteins of a subject, and one or more VHH each directed against one or more target molecules wherein the VHHs belong to the traditional class of *Camelidae* single domain heavy chain antibodies. The present invention also relates to a polypeptide construct comprising one or more VHH each directed against one or more serums protein of a subject, and one or more VHH each directed against one or more target molecules wherein the VHHs belong to a class of *Camelidae* single domain heavy chain antibodies that have human-like sequences. A VHH sequence represented by SEQ ID NO: 19 which binds to TNF-alpha, belongs to this class of VHH peptides. As such, peptides belonging to this class show a high amino acid sequence homology to human VH framework regions and said peptides might be administered to patients directly without expectation of an unwanted immune response therefrom, and without the burden of further humanization.

A subject as used herein is any mammal having a circulatory system in which the fluid therein comprises serum proteins. Examples of circulatory system include blood and lymphatic

systems. Examples of animals include, but are not limited to, rabbits, humans, goats, mice, rats, cows, calves, camels, llamas, monkeys, donkeys, guinea pigs, chickens, sheep, dogs, cats, horses etc.

One embodiment of the present invention is a heterospecific polypeptide construct comprising one or more VHHs each directed against one or more therapeutic and/or diagnostic targets and one or more VHH each directed against one or more serum proteins or polypeptides. As already mentioned, the anti-target VHHs may have the same sequence. Alternatively, the anti-target VHHs may not all have the same sequence, but are directed against the same epitope or different epitopes on the same target, fragments thereof, or antigen thereof. Similarly, the anti-serum protein VHHs may have the same sequence. Alternatively, anti-serum protein VHHs may not all have the same sequence, but are directed against the same epitope or different epitopes on the same serum protein, fragments thereof, or antigen thereof.

In another embodiment of the present invention, the anti-target VHHs may be directed more than one target (*e.g.* vWF and collagen). Similarly, the anti-serum protein VHHs may be directed against more than one serum protein (*e.g.* serum albumin and fibrinogen).

The constructs disclosed herein retain the advantageous properties of VHHs and have a prolonged lifetime in the circulation of an individual. Thus, such constructs are able to circulate in the subject's serum for several days, reducing the frequency of treatment, the inconvenience to the subject and resulting in a decreased cost of treatment.

According to an aspect of the invention a single domain antibody used to form a heterospecific polypeptide construct may be a complete single domain antibody (e.g. a VHH) or a homologous sequence thereof. According to another aspect of the invention, a single domain antibody used to form the heterospecific polypeptide construct may be a functional portion of a complete single domain antibody. According to another aspect of the invention, a single domain antibody used to form the heterospecific polypeptide construct may be a homologous sequence of a complete single domain antibody. According to another aspect of the invention, a single domain antibody used to form the heterospecific polypeptide construct may be a functional portion of a homologous sequence of a complete single domain antibody.

As used herein, an homologous sequence of the present invention may comprise additions, deletions or substitutions of one or more amino acids, which do not substantially alter the functional characteristics of the polypeptides of the invention. The number of amino acid deletions or substitutions is preferably up to 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 41, 42, 43, 44, 45, 46, 47, 48, 49, 50, 51, 52, 53, 54, 55, 56, 57, 58, 59, 60, 61, 62, 63, 64, 65, 66, 67, 68, 69 or 70 amino acids.

A homologous sequence of the present invention may include a polypeptide of the invention which has been humanised.

By humanised is meant mutated so that immunogenicity upon administration in human patients is minor or nonexistent. Humanising a polypeptide, according to the present invention, comprises a step of replacing one or more of the *Camelidae* amino acids by their human counterpart as found in the human consensus sequence, without that polypeptide losing its typical character, *i.e.* the humanisation does not significantly affect the antigen binding capacity of the resulting polypeptide. Such methods are known by the skilled addressee. A humanisation technique may also be performed by a method comprising at least the replacement of the hallmark amino acid at position 45, possibly combined with a replacement of one or more of the other hallmark amino acids at position 37, 44 and 47; numbering according to the Kabat numbering.

Some VHH sequences display a high sequence homology to human VH framework regions and therefore said VHH might be administered to patients directly without expectation of an immune response therefrom, and without the additional burden of humanisation. Therefore, one aspect of the present invention allows for the formation of a heterospecific polypeptide construct without humanisation of the VHH, when said VHH exhibit high homology to human VH framework regions.

A homologous sequence of the present invention may include a sequence of the invention which in another *Camelidae* species such as, for example, camel, dromedary, alpaca, guanaco etc.

Where homologous sequence indicates sequence identity, it means a sequence which presents a high sequence identity (more than 70%, 75%, 80%, 85%, 90%, 95% or 98% sequence identity) with a polypeptide of the invention, and is preferably characterised by similar properties of the parent sequence, namely affinity, said identity calculated using known methods.

A homologous sequence according to the present invention may refer to nucleotide sequences of more than 50, 100, 200, 300, 400, 500, 600, 800 or 1000 nucleotides able to hybridise to the reverse-complement of the nucleotide sequence capable of encoding a native sequence under stringent hybridisation conditions (such as the ones described by SAMBROOK *et al.*, Molecular Cloning, Laboratory Manuel, Cold Spring, Harbor Laboratory press, New York).

As used herein, a functional portion refers to a polypeptide of sufficient size to sufficient size such that the interaction of interest is maintained with affinity of 1×10^{-6} M or better.

Alternatively a functional portion of a polypeptide of the invention comprises a partial deletion of the complete amino acid sequence and still maintains the binding site(s) and protein domain(s) necessary for the binding of and interaction with the target or serum protein.

As used herein, a functional portion of a polypeptide of the invention refers to less than 100% of the sequence (*e.g.*, 99%, 90%, 80%, 70%, 60% 50% etc.), but comprising 5 or more amino acids or 15 or more nucleotides.

A portion of a polypeptide of the invention refers to less than 100% of the sequence (*e.g.*, 99%, 90%, 80%, 70%, 60% 50% etc.), but comprising 5 or more amino acids or 15 or more nucleotides.

A fragment as it refers to a target of the invention refers to less than 100% of the sequence (*e.g.*, 99%, 90%, 80%, 70%, 60% 50% etc.), but comprising 5 or more amino acids or 15 or more nucleotides. A fragment is a sequence of target capable of eliciting an immunogenic

response. It refers to less than 100% of the sequence (*e.g.*, 99%, 90%, 80%, 70%, 60% 50%, etc.), but comprising 5 or more amino acids or 15 or more nucleotides.

The serum protein may be any suitable protein found in the serum of subject, or fragment thereof. In one aspect of the invention, the serum protein is serum albumin, serum immunoglobulins, thyroxine-binding protein, transferrin, or fibrinogen. Depending on the intended use such as the required half-life for effective treatment and/or compartimentalisation of the target antigen, the VHH-partner can be directed to one of the above serum proteins.

The heterospecific polypeptide constructs disclosed herein may be made by the skilled artisan according to methods known in the art or any future method. For example, the VHHs may be obtained using methods known in the art such as by immunising a camel and obtaining hybridomas therefrom, or cloning the VHHs using molecular biology techniques known in the art and subsequent selection by using phage display.

The anti-serum protein single domain antibody may be directed against a polypeptide of a serum protein or a whole protein. The anti-target single domain antibody may be directed against a polypeptide of said target of the whole target. Methods for scanning a protein for immunogenic polypeptides are well known in the art.

The single domain antibodies may be joined using methods known in the art or any future method. For example, they may be fused by chemical cross-linking by reacting aminoacid residues with an organic derivatising agent such as described by Blattler *et al*, Biochemistry 24,1517-1524; EP294703. Alternatively, the single domain antibody may be fused genetically at the DNA level *i.e.* a polynucleotide construct formed which encodes the complete polypeptide construct comprising one or more anti-target single domain antibodies and one or more anti-serum protein single domain antibodies. A method for producing bivalent or multivalent VHH polypeptide constructs is disclosed in PCT patent application WO 96/34103. One way of joining multiple single domain antibodies is via the genetic route by linking single domain antibody coding sequences either directly or *via* a peptide linker. For example, the C-terminal end of the first single domain antibody may be linked to the N-terminal end of the next single domain antibody. This linking mode can be extended in order to link additional

single domain antibodies for the construction and production of tri-, tetra-, etc. functional constructs.

Delivery

One embodiment of the present invention is a heterospecific polypeptide construct as disclosed herein for use in treating, preventing and/or alleviating the symptoms of disorders requiring the delivery of a therapeutic compound that is able pass through the gastric environment without being inactivated. As known by persons skilled in the art, once in possession of said heterospecific polypeptide construct, formulation technology may be applied to release a maximum amount of heterospecific polypeptide construct in the right location (in the stomach, in the colon, etc.). This method of delivery is important for treating, prevent and/or alleviate the symptoms of disorder whose targets that are located in the gut system. An aspect of the invention is a method for treating, preventing and/or alleviating the symptoms of a disorder requiring the delivery of a therapeutic compound that is able pass through the gastric environment without being inactivated, by orally administering to a subject a heterospecific polypeptide construct specific for a target related to the disorder.

Another embodiment of the present invention is a heterospecific polypeptide construct for use in treating, preventing and/or alleviating the symptoms of disorders requiring the delivery of a therapeutic compound to the vaginal and/or rectal tract. In a non-limiting example, a formulation according to the invention comprises a heterospecific polypeptide construct directed against one or more targets in the form of a gel, cream, suppository, film, or in the form of a sponge or as a vaginal ring that slowly releases the active ingredient over time. An aspect of the invention is a method for treating, preventing and/or alleviating the symptoms of disorders requiring the delivery of a therapeutic compound to the vaginal and/or rectal tract, by vaginally and/or rectally administering to a subject a heterospecific polypeptide construct specific for a target related to the disorder.

Another embodiment of the present invention is a heterospecific polypeptide construct for use in treating, preventing and/or alleviating the symptoms of disorders requiring the delivery of a therapeutic compound to the upper respiratory tract and lung. In a non-limiting example, a formulation according to the invention, comprises a heterospecific polypeptide construct directed against one or more targets in the form of a nasal spray (*e.g.* an aerosol). Since a

heterospecific polypeptide construct are small, they can reach their target much more effectively than therapeutic IgG molecules. An aspect of the invention is a method for treating, preventing and/or alleviating the symptoms of disorders requiring the delivery of a therapeutic compound to the upper respiratory tract and lung, by administering to a subject said heterospecific polypeptide construct specific for a target related to the disorder by inhalation.

One embodiment of the present invention is a heterospecific polypeptide construct for use in treating, preventing and/or alleviating the symptoms of disorders wherein the permeability of the intestinal mucosa is increased. Because of their small size, a heterospecific polypeptide construct can pass through the intestinal mucosa and reach the bloodstream more efficiently in subjects suffering from disorders which cause an increase in the permeability of the intestinal mucosa, for example Crohn's disease. An aspect of the invention is a method for treating, preventing and/or alleviating the symptoms of disorders wherein the permeability of the intestinal mucosa is increased, by orally administering to a subject a heterospecific polypeptide construct specific for a target related to the disorder.

One embodiment of the present invention is a heterospecific polypeptide construct for use in treating, preventing and/or alleviating the symptoms of disorders requiring the delivery of a therapeutic compound that is able pass through the tissues beneath the tongue effectively. A formulation of said heterospecific polypeptide construct, for example, a tablet, spray, drop is placed under the tongue and adsorbed through the mucus membranes into the capilliary network under the tongue. An aspect of the invention is a method for treating, preventing and/or alleviating the symptoms of disorders requiring the delivery of a therapeutic compound that is able pass through the tissues beneath the tongue effectively, by sublingually administering to a subject a heterospecific polypeptide construct specific for a target related to the disorder.

One embodiment of the present invention is a heterospecific polypeptide construct for use in treating, preventing and/or alleviating the symptoms of disorders requiring the delivery of a therapeutic compound that is able pass through the skin effectively. A formulation of said heterospecific polypeptide construct, for example, a cream, film, spray, drop, patch, is placed on the skin and passes through. An aspect of the invention is a method for treating, preventing and/or alleviating the symptoms of disorders requiring the delivery of a therapeutic

compound that is able pass through the skin effectively, by topically administering to a subject a heterospecific polypeptide construct specific for an target related to the disorder.

One embodiment of the present invention is a heterospecific polypeptide construct for use in treating, preventing and/or alleviating the symptoms of disorders requiring the delivery of a therapeutic compound intraveneously. An aspect of the invention is a method for treating, preventing and/or alleviating the symptoms of disorders requiring the delivery of a therapeutic compound via the bloodstream.

General target

Another embodiment of the present invention is a heterospecific polypeptide construct as disclosed herein for use in treating, preventing and/or alleviating the symptoms of a disorder requiring a therapeutic or diagnostic compound which is not rapidly cleared from the circulation. An aspect of the invention is the use of a said construct for the preparation of a medicament for treating, preventing and/or alleviating the symptoms of a disorder requiring a therapeutic or diagnostic compound which is not rapidly cleared from the circulation. Another aspect of the invention is a method for treating, preventing and/or alleviating the symptoms of a disorder requiring a therapeutic or diagnostic compound which is not rapidly cleared from the circulation by administering a heterospecific polypeptide construct as disclosed herein to an individual. According to the present invention, the anti-target VHH of said heterospecific polypeptide is directed against a target involved in a cause or a manifestation of said disorder, or involved in causing symptoms thereof. By using a heterospecific polypeptide construct of the present invention to treat or diagnose an aforementioned disorder, the depletion of said construct is retarded.

Another embodiment of the present invention is a heterospecific polypeptide construct as disclosed herein for use in treating, preventing and/or alleviating the symptoms of a disorder requiring a therapeutic or diagnostic compound which remains active in the circulation for extended periods of time. An aspect of the invention is the use of said construct for the preparation of a medicament for treating, preventing and/or alleviating the symptoms of a disorder requiring a therapeutic or diagnostic compound which remains active in the circulation for extended periods of time. Another aspect of the invention is a method for treating, preventing and/or alleviating the symptoms of a disorder requiring a therapeutic or diagnostic compound that is able to circulate in the patients serum for several days, by

administering a heterospecific polypeptide construct as disclosed herein to an individual. According to the present invention, the anti-target VHH of said heterospecific polypeptide is directed against a target involved in a cause or a manifestation of said disorder, or involved in causing symptoms thereof. By using a heterospecific polypeptide construct of the present invention to treat or diagnose an aforementioned disorder, the frequency of treatment is reduced, so resulting in a decreased cost of treatment.

Another embodiment of the present invention is a heterospecific polypeptide construct as disclosed herein for use in treating, preventing and/or alleviating the symptoms of a disorder relating to allergies. An aspect of the invention is the use of said construct for the preparation of a medicament for treating, preventing and/or alleviating the symptoms of a disorder relating to allergies. Another aspect of the invention is a method for treating, preventing and/or alleviating the symptoms of a disorder relating to allergies, by administering a heterospecific polypeptide construct as disclosed herein to an individual. According to the present invention, the anti-target VHH of said heterospecific polypeptide is directed against a target involved in a cause or a manifestation of said disorder, or involved in causing symptoms thereof.

The above aspects and embodiments of the invention also apply when an anti-serum single domain antibody of the aforementioned heterospecific polypeptide constructs corresponds to a sequence represented by SEQ ID NOs: 1 to 3, a homologous sequence thereof, a functional portion thereof, or a homologous sequence of a functional portion.

The above aspects and embodiments of the invention also apply when an anti-target single domain antibody of the aforementioned heterospecific polypeptide constructs corresponds to a sequence represented by SEQ ID NOs: 17 to 29, a homologous sequence thereof, a functional portion thereof, or a homologous sequence of a functional portion, said target being TNF-alpha.

The above aspects and embodiments of the invention also apply when an anti-target single domain antibody of the aforementioned heterospecific polypeptide constructs corresponds to a sequence represented by SEQ ID NOs: 30 to 39 a homologous sequence thereof, a functional portion thereof, or a homologous sequence of a functional portion, said target being vWF.

The above aspects and embodiments of the invention also apply when an anti-target single domain antibody of the aforementioned heterospecific polypeptide constructs correspond to a sequence represented by SEQ ID NOs: 7 to 16 a homologous sequence thereof, a functional portion thereof, or a homologous sequence of a functional portion, said target being IgE.

The above aspects and embodiments of the invention also apply when an anti-target single domain antibody of the aforementioned heterospecific polypeptide construct corresponds to a sequence of an anti-IFN0gamma VHH, a homologous sequence thereof, a functional portion thereof, or a homologous sequence of a functional portion.

The above aspects and embodiments of the invention also apply when the aforementioned heterospecific polypeptide construct corresponds to a sequence represented by any of SEQ ID NOs: 4 to 6, a homologous sequence thereof, a functional portion thereof, or a homologous sequence of a functional portion thereof.

A non-limiting example, in relation to allergies, of a target against which an anti-target single domain antibody may be directed is IgE. During their lifetime, several subjects develop an allergic response to harmless parasites such as *Dermatophagoides pteronyssinus*, the house dust mite or to substances such as clumps, plastics, metals. This results in an induction of IgE molecules that initiates a cascade of immunological responses. One aspect of the present invention is a heterospecific polypeptide construct comprising one or more anti-IgE single domain antibodies fused to one or more anti-serum protein single domain antibodies. In one aspect of the invention, said anti-IgE single domain antibodies prevents the interaction of IgE with their receptor(s) on mast cells and basophils, so blocking initiation of the immunological cascade and a subsequent allergic reaction. In another aspect an anti-serum protein single domain antibody is directed to one of the subject's serum proteins. A heterospecific polypeptide construct as disclosed herein thus reduces or prevents an allergic response due to common or unusual allergens. Furthermore, the construct has a prolonged lifetime in the blood so increasing the therapeutic window.

TNF-alpha

Another embodiment of the present invention is a heterospecific polypeptide construct as disclosed herein for use in treating, preventing and/or alleviating the symptoms of a disorder mediated by inflammatory molecules. An aspect of the invention is the use of said construct for the preparation of a medicament for treating, preventing and/or alleviating the symptoms of a disorder mediated by inflammatory molecules. Another aspect of the invention is a method for treating, preventing and/or alleviating the symptoms of a disorder mediated by inflammatory molecules, by administering a heterospecific polypeptide construct as disclosed herein to an individual. According to the present invention, an anti-target single domain antibody of said heterospecific polypeptide is directed against a target involved in a cause or a manifestation of said disorder, or involved in causing symptoms thereof.

According to one aspect of the invention, a target against which a single domain antibody of a heterospecific polypeptide construct is directed is tumor necrosis factor alpha (TNF-alpha). TNF-alpha is believed to play an important role in various disorders, for example in inflammatory disorders such as rheumatoid arthritis, Crohn's disease, ulcerative colitis and multiple sclerosis. Anti-target single domain antibodies may be directed against whole TNF-alpha or a fragment thereof, or a fragment of a homologous sequence thereof.

One aspect of the present invention relates to a heterospecific polypeptide construct comprising one or more anti-TNF-alpha single domain antibody fused to one or more anti-serum protein single domain antibody, the sequences of said anti-TNF-alpha single domain antibody corresponding to any of SEQ ID NOs: 17 to 29, derived from *Camelidae* heavy chain antibodies (VHHs), which bind to TNF-alpha.

The aspect of the invention relating to an anti-TNF-alpha single domain antibody is not limited to a polypeptide represented by SEQ ID NOs: 17 to 29, but may be extended to encompass polypeptides comprising *Camelidae* antibodies of any class directed towards TNF-alpha. Any of the heterospecific polypeptide constructs disclosed herein may comprise a *Camelidae* antibody of any class, of the regular class or of the class of human-like *Camelidae* antibodies as disclosed herein. These antibodies include the full length *Camelidae* antibodies, domains and may comprise a human Fc domain if effector functions are needed.

One embodiment of the present invention is a heterospecific polypeptide construct comprising one or more anti-TNF-alpha single domain antibodies fused to one or more anti-serum protein single domain antibodies for use in treating, preventing and/or alleviating the symptoms of inflammatory disorders. TNF-alpha is involved in inflammatory processes, and the blocking of TNF-alpha action can have an anti-inflammatory effect, which is highly desirable in certain disorder states such as, for example, Crohn's disease. Oral delivery of these heterospecific polypeptide construct results in the delivery of such molecules in an active form in the colon at sites that are affected by the disorder. These sites are highly inflamed and contain TNF-alpha producing cells. These heterospecific polypeptide constructs can neutralise the TNF-alpha locally, avoiding distribution throughout the whole body and thus limiting negative side-effects. Genetically modified microorganisms such as *Micrococcus lactis* are able to secrete antibody fragments. Such modified microorganisms can be used as vehicles for local production and delivery of antibody fragments in the intestine. By using a strain which produces a TNF-alpha neutralising antibody fragment, inflammatory bowel disorder could be treated. Another aspect of the invention is a heterospecific polypeptide construct comprising one or more anti-TNF-alpha single domain antibodies fused to one or more anti-serum protein single domain antibodies for use in the treatment, prevention and/or alleviation of disorders relating to inflammatory processes, wherein said heterospecific polypeptide construct is administered intravenously, orally, sublingually, topically, nasally, vaginally, rectally or by inhalation. Another aspect of the invention is the use of a heterospecific polypeptide construct comprising one or more anti-TNF-alpha single domain antibodies fused to one or more anti-serum protein single domain antibodies for the preparation of a medicament for the treatment, prevention and/or alleviation of disorders relating to inflammatory processes, wherein said heterospecific polypeptide construct is administered intravenously, orally, sublingually, topically, nasally, vaginally, rectally or by inhalation. Another aspect of the invention is a method of treating, preventing and/or alleviating disorders relating to inflammatory processes, comprising administering to a subject a heterospecific polypeptide construct comprising one or more anti-TNF-alpha single domain antibodies fused to one or more anti-serum protein single domain antibodies intravenously, orally, sublingually, topically, nasally, vaginally, rectally or by inhalation. Another aspect of the invention is a heterospecific polypeptide construct comprising one or more anti-TNF-alpha single domain antibodies fused to one or more anti-serum protein single domain antibodies

for use in the treatment, prevention and/or alleviation of disorders relating to inflammatory processes. Another aspect of the invention is a heterospecific polypeptide construct comprising one or more anti-TNF-alpha single domain antibodies fused to one or more anti-serum protein single domain antibodies for the preparation of a medicament for the treatment, prevention and/or alleviation of disorders relating to inflammatory processes. Anti-TNF-alpha single domain antibodies of the present invention may be derived from a class of VHHs with high homology to the human VH sequence, or may be derived from any of the other classes of VHHs, including the major class of VHH.

The above aspects and embodiments apply to a heterospecific polypeptide construct comprising one or more anti-TNF-alpha single domain antibodies fused to one or more anti-serum protein single domain antibodies wherein said anti-TNF single domain antibodies correspond to any of SEQ ID NOs: 17 to 29 (anti-TNF-alpha VHHs), a homologous sequence thereof, a functional portion thereof, of a homologous sequence of a functional portion thereof.

The above aspects and embodiments apply to a heterospecific polypeptide construct comprising one or more anti-TNF-alpha single domain antibodies fused to one or more anti-serum protein single domain antibodies wherein said anti-serum protein single domain antibodies correspond to any of SEQ ID NOs: 1 to 3 (anti-serum protein VHHs), a homologous sequence thereof, a functional portion thereof, of a homologous sequence of a functional portion thereof.

The above aspects and embodiments apply to a heterospecific polypeptide construct comprising a sequence corresponding to any of SEQ ID NOs: 4 to 6 (anti-TNF-alpha VHH/anti-serum albumin VHH), a homologous sequence thereof, a functional portion thereof, a homologous sequence of a functional portion thereof. The inventors have found that a heterospecific polypeptide construct comprising a sequence corresponding to any of SEQ ID NOs: 4 to 6 surprisingly exhibits higher than expected affinity towards its target and prolonged half-life in the circulatory system.

vWF

Platelet-mediated aggregation is the process wherein von Willebrand Factor (vWF)-bound collagen adheres to platelets and/or platelet receptors (examples of both are gpIa/IIa, gpIb, or collagen), ultimately resulting in platelet activation. Platelet activation leads to fibrinogen binding, and finally to platelet aggregation. It is another aspect of the present invention to provide heterospecific polypeptide constructs which modulate processes which comprise platelet-mediated aggregation such as, for example, vWF-collagen binding, vWF-platelet receptor adhesion, collagen-platelet receptor adhesion, platelet activation, fibrinogen binding and/or platelet aggregation. Said heterospecific polypeptide constructs are derived from single domain antibodies directed towards vWF, vWF A1 or A3 domains or collagen. Said antibodies may be directed against whole vWF, vWF A1 or A3 domains or collagen or a fragment thereof, or a fragment of a homologous sequence thereof.

According to one aspect of the invention, a target against which a heterospecific polypeptide construct comprising one or more anti-TNF-target single domain antibodies fused to one or more anti-serum protein single domain antibodies is directed is von Willebrand factor (vWF). According to one aspect of the invention, the target is vWF A1 or A3 domains. According to another aspect of the invention, the target is gpIb. According to one aspect of the invention, the target is gpIa/IIA. According to one aspect of the invention, the target is collagen.

One aspect of the present invention relates a heterospecific polypeptide construct comprising one or more anti-vWF single domain antibodies fused to one or more anti-serum protein VHHs, the sequences of said anti-vWF single domain antibodies corresponding to any of SEQ ID NOs: 30 to 39, derived from *Camelidae* heavy chain antibodies (VHHs), which bind to vWF.

The aspect of the invention relating to anti-vWF, anti-vWF A1 or anti-vWF A3, anti-gpIb, anti-gpIa/IIa, or anti-collagen VHHs is not limited to polypeptides represented by SEQ ID NOs: 30 to 39, but may be extended to encompass polypeptides comprising *Camelidae* antibodies of any class directed towards vWF, vWF A1 or A3 domains or collagen. The VHHs disclosed herein may comprise a *Camelidae* antibody of any class, of the traditional class or of the class of human-like *Camelidae* antibodies as disclosed herein. These polypeptides include

the full length *Camelidae* antibodies, and may comprise a human Fc domain if effector functions are needed.

One embodiment of the present invention is a heterospecific polypeptide construct comprising one or more anti-target single domain antibodies fused to one or more anti-serum protein single domain antibodies target, wherein the target is any of vWF, vWF A1 or A3 domains or collagen for use in treating, preventing and/or alleviating the symptoms of disorders or conditions relating to platelet-mediated aggregation or dysfunction thereof. Said disorders include transient cerebral ischemic attack, unstable angina pectoris, cerebral infarction, myocardial infarction, peripheral arterial occlusive disease, restenosis. Said conditions include those arising from coronary by-pass graft, coronary artery valve replacement and coronary interventions such angioplasty, stenting, or atherectomy. One aspect of the invention is a heterospecific polypeptide construct comprising one or more anti-target single domain antibodies fused to one or more anti-serum protein single domain antibodies, wherein the target is any of vWF, vWF A1 or A3 domains or collagen for use in the treatment, prevention and/or alleviation of disorders or conditions relating to platelet-mediated aggregation or dysfunction thereof, wherein said heterospecific polypeptide construct is administered intravenously, orally, sublingually, topically, nasally, vaginally, rectally or by inhalation. Another aspect of the invention is the use of a heterospecific polypeptide construct comprising one or more anti-target single domain antibodies fused to one or more anti-serum protein single domain antibodies target, wherein the target is any of vWF, vWF A1 or A3 domains or collagen for the preparation of a medicament for the treatment, prevention and/or alleviation of disorders or conditions relating to platelet-mediated aggregation or dysfunction thereof, wherein said heterospecific polypeptide construct is administered intravenously, orally, sublingually, topically, nasally, vaginally, rectally or by inhalation. Another aspect of the invention is a method of treating, preventing and/or alleviating disorders or conditions relating to relating to platelet-mediated aggregation or dysfunction thereof, comprising administering to a subject a heterospecific polypeptide construct comprising one or more anti-target single domain antibodies fused to one or more anti-serum protein single domain antibodies target, wherein the target is any of vWF, vWF A1 or A3 domains or collagen, wherein said heterospecific polypeptide construct is administered intravenously, orally, sublingually, topically, nasally, vaginally, rectally or by inhalation. Another aspect of the invention is a heterospecific polypeptide construct comprising one or

more anti-target single domain antibodies fused to one or more anti-serum protein single domain antibodies, wherein the target is any of vWF, vWF A1 or A3 domains or collagen for use in the treatment, prevention and/or alleviation of disorders or conditions relating to platelet-mediated aggregation or dysfunction thereof. Another aspect of the invention is a use of a heterospecific polypeptide construct comprising one or more anti-target single domain antibodies fused to one or more anti-serum protein single domain antibodies, wherein the target is any of vWF, vWF A1 or A3 domains or collagen for the preparation of a medicament for the treatment, prevention and/or alleviation of disorders or conditions relating to platelet-mediated aggregation or dysfunction thereof. Anti-vWF, anti-vWF A1 or anti-vWF A3 or anti-collagen VHHs of the present invention may be derived from the class of VHHs with high homology to the human VH sequence, or may be derived from any of the other classes of VHHs, including the major class of VHH.

The above aspects and embodiments apply to a heterospecific polypeptide construct comprising one or more anti-vWF single domain antibodies wherein said single domain antibodies correspond to any of SEQ ID NOs: 30 to 39 (anti-vWF VHHs), a homologous sequence thereof, a functional portion thereof, of a homologous sequence of a functional portion thereof.

The above aspects and embodiments apply to a heterospecific polypeptide construct comprising one or more anti-target single domain antibodies fused to one or more anti-serum protein single domain antibodies, wherein the target is any of vWF, vWF A1 or A3 domains or collagen and wherein said anti-serum protein single domain antibodies correspond to any of SEQ ID NOs: 1 to 3, a homologous sequence thereof, a functional portion thereof, of a homologous sequence of a functional portion thereof.

The above aspects and embodiments apply to a heterospecific polypeptide construct corresponding to any of SEQ ID NO: 40 to 42 (anti-vWF VHH/anti-serum albumin VHH), a homologous sequence thereof, a functional portion thereof, of a homologous sequence of a functional portion thereof.

IgE

During their lifetime, subjects may develop an allergic response to harmless parasites (*e.g. Dermatophagoides pteronyssinus*, house dust mite) or substances (clumps, plastics, metals). This results in the induction of IgE molecules that initiate a cascade of immunological responses. One aspect of the present invention a heterospecific polypeptide construct comprising one or more IgE-specific single domain antibodies, said heterospecific polypeptide construct preventing the interaction of IgEs with their receptor(s) on mast cells and basophils. As such they prevent the initiation of the immunological cascade, an allergic reaction.

According to one aspect of the invention, a target against which a heterospecific polypeptide construct comprising one or more anti-target single domain antibodies fused to one or more anti-serum protein single domain antibodies is directed is IgE. Said antibodies may be directed against whole IgE or a fragment thereof, or a fragment of a homologous sequence thereof.

One aspect of the present invention relates to a heterospecific polypeptide construct comprising one or more anti-IgE single domain antibodies fused to one or more anti-serum protein single domain antibodies, the sequences of said anti-IgE single domain antibodies corresponding to any of SEQ ID NOs: 7 to 16, derived from *Camelidae* heavy chain antibodies (VHHs), which bind to IgE.

An aspect of the invention relating to anti-IgE VHHs is not limited to polypeptides represented by SEQ ID NOs: 7 to 16, but may be extended to encompass polypeptides comprising *Camelidae* antibodies of any class directed towards IgE. The VHHs disclosed herein may comprise a *Camelidae* antibody of any class, of the traditional class or of the class of human-like *Camelidae* antibodies as disclosed herein. These polypeptides include the full length *Camelidae* antibodies, and may comprise a human Fc domain if effector functions are needed.

One embodiment of the present invention is a heterospecific polypeptide construct comprising one or more anti-IgE single domain antibodies fused to one or more anti-serum

protein single domain antibodies for use in treating, preventing and/or alleviating the symptoms of disorders relating to allergies. Said disorders comprise a wide range of IgE-mediated diseases such as hay fever, asthma, atopic dermatitis, allergic skin reactions, allergic eye reactions and food allergies. One aspect of the invention is a heterospecific polypeptide construct comprising one or more anti-IgE single domain antibodies fused to one or more anti-serum protein single domain antibodies for use in the treatment, prevention and/or alleviation of disorders relating to allergies, wherein said VHH is administered intravenously, orally, sublingually, topically, nasally, vaginally, rectally or by inhalation. Another aspect of the invention is the use of a heterospecific polypeptide construct comprising one or more anti-IgE single domain antibodies fused to one or more anti-serum protein single domain antibodies for the preparation of a medicament for the treatment, prevention and/or alleviation of disorders relating to allergies, wherein said heterospecific polypeptide construct is administered intravenously, orally, sublingually, topically, nasally, vaginally, rectally or by inhalation. Another aspect of the invention is a method of treating, preventing and/or alleviating disorders relating to allergies, comprising administering to a subject a heterospecific polypeptide construct comprising one or more anti-IgE single domain antibodies fused to one or more anti-serum protein single domain antibodies intravenously, orally, sublingually, topically, nasally, vaginally, rectally or by inhalation. Another aspect of the invention is a heterospecific polypeptide construct comprising one or more anti-IgE single domain antibodies fused to one or more anti-serum protein single domain antibodies for use in the preparation of a medicament for the treatment, prevention and/or alleviation of disorders relating to allergies. Another aspect of the invention is a use of a heterospecific polypeptide construct comprising one or more anti-IgE single domain antibodies fused to one or more anti-serum protein single domain antibodies for the preparation of a medicament for the treatment, prevention and/or alleviation of disorders relating to allergies. Anti-IgE VHHs of the present invention may be derived from the new class of VHHs described above, or may be derived from any of the other classes of VHHs, including the major class of VHH.

The above aspects and embodiments apply to a heterospecific polypeptide construct comprising one or more anti-IgE single domain antibodies fused to one or more anti-serum protein single domain antibodies, wherein anti IgE- single domain antibodies correspond to any of SEQ ID NOs: 7 to 16 (anti-IgE VHHs), a homologous sequence thereof, a functional portion thereof, of a homologous sequence of a functional portion thereof.

The above aspects and embodiments apply to a heterospecific polypeptide construct comprising one or more anti-IgE single domain antibodies fused to one or more anti-serum protein single domain antibodies wherein said anti-serum protein single domain antibodies correspond to any of SEQ ID NOs: 1 to 3 (anti-protein serum VHHs), a homologous sequence thereof, a functional portion thereof, of a homologous sequence of a functional portion thereof.

The above aspect and embodiments apply to a heterospecific polypeptide construct corresponding to any of SEQ ID NOs: 43 to 45 (anti-IgE VHH/anti-serum albumin VHH), a homologous sequence thereof, a functional portion thereof, of a homologous sequence of a functional portion.

A heterospecific polypeptide construct as disclosed herein prevents thus reduces or prevents an allergic response due to common or unusual allergens. Furthermore, the construct has a prolonged lifetime in the blood so increasing the therapeutic window.

IFN-gamma

According to one aspect of the invention, a target against which one or more anti-target single domain antibodies of a heterospecific polypeptide construct comprising one or more anti-target single domain antibodies fused to one or more anti-serum protein single domain antibodies is directed against is interferon-gamma (IFN-gamma). IFN-gamma is secreted by some T cells. In addition to its anti-viral activity, IFN-gamma stimulates natural killer (NK) cells and T helper 1 (Th1) cells, and activates macrophages and stimulates the expression of MHC molecules on the surface of cells. Hence, IFN-gamma generally serves to enhance many aspects of immune function, and is a candidate for treatment of disorders where the immune system is over-active *e.g.* Crohn's disease, autoimmune disorders and organ plant rejection in addition inflammatory disorders such as rheumatoid arthritis, Crohn's disease, ulcerative colitis and multiple sclerosis.

One aspect of the present invention relates to a heterospecific polypeptide construct comprising one or more anti-IFN-gamma single domain antibodies fused to one or more anti-serum protein single domain antibodies, the sequences of said anti-IFN-gamma single

domain antibodies corresponding to any of SEQ ID NOs: 46 to 62 derived from *Camelidae* heavy chain antibodies (VHHs), which bind to IFN-gamma.

The aspect of the invention relating to anti-IFN-gamma VHH is not limited to polypeptides represented by SEQ ID NOs: 46 to 62, but may be extended to encompass polypeptides comprising *Camelidae* antibodies of any class directed towards IFN-gamma. Any of the polypeptides disclosed herein may comprise a *Camelidae* antibody of any class, of the traditional class or of the new class of human-like *Camelidae* antibodies as disclosed herein. Said antibodies may be directed against whole IFN-gamma or a fragment thereof, or a fragment of a homologous sequence thereof. These polypeptides include the full length *Camelidae* antibodies, and may comprise a human Fc domain if effector functions are needed.

One embodiment of the present invention is a heterospecific polypeptide construct comprising one or more anti-IFN-gamma single domain antibodies fused to one or more anti-serum protein single domain antibodies for use in treating, preventing and/or alleviating the symptoms of the disorders wherein the immune system is overactive, as mentioned above. Current therapy consists of intravenous administration of anti-IFN-gamma antibodies. Oral delivery of these heterospecific polypeptide constructs results in the delivery of such molecules in an active form in the colon at sites that are affected by the disorder. These sites are highly inflamed and contain IFN-gamma producing cells. These heterospecific polypeptide constructs can neutralise the IFN-gamma locally, avoiding distribution throughout the whole body and thus limiting negative side-effects. Genetically modified microorganisms such as *Micrococcus lactis* are able to secrete antibody fragments. Such modified microorganisms can be used as vehicles for local production and delivery of antibody fragments in the intestine. By using a strain which produces a IFN-gamma neutralising antibody fragment, inflammatory bowel disorder could be treated. Another aspect of the invention is a heterospecific polypeptide construct comprising one or more anti-IFN-gamma single domain antibodies fused to one or more anti-serum protein single domain antibodies for use in the treatment, prevention and/or alleviation of disorders wherein the immune system is overactive, wherein said heterospecific polypeptide construct is administered intravenously, orally, sublingually, topically, nasally, vaginally, rectally or by inhalation. Another aspect of the invention is the use of a heterospecific polypeptide construct

comprising one or more anti-IFN-gamma single domain antibodies fused to one or more anti-serum protein single domain antibodies for the preparation of a medicament for the treatment, prevention and/or alleviation of disorders wherein the immune system is over active, wherein said heterospecific polypeptide construct is administered intravenously, orally, sublingually, topically, nasally, vaginally, rectally or by inhalation. Another aspect of the invention is a method of treating, preventing and/or alleviating disorders wherein the immune system is overactive, comprising administering to a subject a heterospecific polypeptide construct comprising one or more anti-IFN-gamma single domain antibodies fused to one or more anti-serum protein single domain antibodies intravenously, orally, sublingually, topically, nasally, vaginally, rectally or by inhalation. Another aspect of the invention is a heterospecific polypeptide construct comprising one or more anti-IFN-gamma single domain antibodies fused to one or more anti-serum protein single domain antibodies for use in the preparation of a medicament for the treatment, prevention and/or alleviation of disorders wherein the immune system is overactive. Another aspect of the invention is a use of a heterospecific polypeptide construct comprising one or more anti-IFN-gamma single domain antibodies fused to one or more anti-serum protein single domain antibodies for use in the preparation of a medicament for the treatment, prevention and/or alleviation of disorders wherein the immune system is over active. Anti-IFN-gamma VHHs of the present invention may be derived from the new class of VHHs described above, or may be derived from any of the other classes of VHHs, including the major class of VHH.

The above aspect and embodiments apply to a heterospecific polypeptide construct comprising one or more anti-IFN-gamma VHHs fused to one or more anti-serum protein single domain antibodies wherein said anti-IFN-gamma VHHs correspond to any of SEQ ID NOs: 46 to 62, a homologous sequence thereof, a functional portion thereof, of a homologous sequence of a functional portion.

The above aspects and embodiments apply to a heterospecific polypeptide construct comprising one or more anti-IFN-gamma single domain antibodies fused to one or more anti-serum protein VHHs wherein said anti-serum protein VHHs correspond to any of SEQ ID NOs: 1 to 3, a homologous sequence thereof, a functional portion thereof, of a homologous sequence of a functional portion thereof.

The above aspects and embodiments apply to a heterospecific polypeptide construct corresponding to any of SEQ ID NOs: 63 to 65 (anti-IFN-gamma VHH/anti-serum albumin VHH), a homologous sequence thereof, a functional portion thereof, of a homologous sequence of a functional portion.

Cloning vehicle

One embodiment of the present invention is a recombinant clone comprising nucleic acid encoding a heterospecific polypeptide construct according to the invention. In one aspect of the invention, said nucleic acid encodes one or more single domain antibodies each directed to a therapeutic or diagnostic target antigen and nucleic acid encoding one or more single domain antibodies directed to a serum protein, said single domain antibodies linked without intervening linkers, or with one or more peptide linker sequences. According to one aspect of the invention, a linker sequence is any suitable linker sequence known in the art. According to another aspect of the invention, a linker sequence is a naturally occurring sequence. Preferred properties of linkers sequences are that they are not immunogenic or not significantly immunogenic, they can provide sufficient flexibility to the heterospecific polypeptide construct, and are resistant to proteolytic degradation. An example of a linker according to the invention is that disclosed in PCT/EP96/01725 which is derived from the hinge region of VHH.

According to another aspect of the invention, a clone comprises nucleic acid of a encoding a polypeptide corresponding to a sequence represented by any of SEQ ID NOs: 1 to 3, a homologous sequence thereof, a functional portion thereof, or a homologous sequence of a functional portion, together with nucleic acid encoding one or more anti-target single domain antibodies, a homologous sequence thereof, a functional portion thereof, or a homologous sequence of a functional portion thereof.

According to another aspect of the invention, a clone comprises nucleic acid capable of encoding a polypeptide corresponding to a sequence represented by any of SEQ ID NOs: 7 to 39, a homologous sequence thereof, a functional portion thereof, or a homologous sequence of a functional portion thereof, together with nucleic acid encoding one or more anti-serum protein single domain antibodies, a homologous sequence thereof, a functional portion thereof, or a homologous sequence of a functional portion thereof.

According to another aspect of the invention, a clone comprises nucleic acid capable of encoding a polypeptide corresponding to a sequence represented by any of SEQ ID NOs: 4 to 6, a homologous sequence thereof, a functional portion thereof, or a homologous sequence of a functional portion thereof.

It is within the scope of the invention that nucleic acid encoding multiple anti-target and/or multiple anti-serum VHHs are present in a clone of the invention.

By transforming a compatible host with a clone encoding a heterospecific polypeptide construct of the invention, the heterospecific polypeptide construct can be produced in sufficient quantities for use in therapy. Examples of organisms into which said clone may be transformed include, but are not limited to *E. coli* or *Sacchoromyces cerevisiae.*

Another embodiment of the present invention is a method for prolonging the half-life of an anti-target-VHH comprising the step of joining thereto one or more anti-serum albumin VHHs. As already mentioned above, methods for joining are known in the art or may be any future method, for example, they may be fused by chemical coupling, fused at the DNA level etc.

Treating, preventing and/or alleviating the symptoms of one or more of the disorders mentioned herein generally involves administering to a subject a "therapeutically effective amount" of polypeptide. By "therapeutically effective amount", "therapeutically effective dose" and "effective amount" means the amount needed to achieve the desired result or results. One of ordinary skill in the art will recognise that the potency and, therefore, an "effective amount" can vary for the various compounds that inhibit a disorder pathway used in the invention. One skilled in the art can readily assess the potency of the compound.

As used herein, the term "compound" refers to a heterospecific polypeptide construct as disclosed herein, a polypeptide represented by SEQ ID NOs: 4 to 6, a homologous sequence thereof, or a homologue thereof, or a nucleic acid capable of encoding said polypeptide.

By "pharmaceutically acceptable" is meant a material that is not biologically or otherwise undesirable, *i.e.*, the material may be administered to an individual along with the compound

without causing any undesirable biological effects or interacting in a deleterious manner with any of the other components of the pharmaceutical composition in which it is contained.

The invention disclosed herein is useful for treating or preventing a condition relating to a disorder as mentioned herein (*e.g.* allergy and/or inflammation), in a subject and comprising administering a pharmaceutically effective amount of a compound or composition that binds to a component involved in the disorder pathway (*e.g.* to IgE and/or TNF-alpha in the blood stream), so inhibiting the disorder pathway and the disorder.

One aspect of the present invention is the use of compounds of the invention for treating or preventing a condition relating to a disorder as mentioned herein (*e.g.* allergy and/or inflammation), in a subject and comprising administering a pharmaceutically effective amount of a compound in combination with another, such as, for example, aspirin.

The present invention is not limited to the administration of formulations comprising a single compound of the invention. It is within the scope of the invention to provide combination treatments wherein a formulation is administered to a patient in need thereof that comprises more than one compound of the invention.

It is well known in the art how to determine the inhibition of a disorder pathway using the standard tests described herein, or using other similar tests. Preferably, the method would result in at least a 10% reduction in an indicator of the disorder, including, for example, 15%, 20%, 25%, 30%, 40%, 50%,60%, 70%, 80%, 90%, 100%, or any amount in between, more preferably by 90%. For example, an inhibition of an allergic pathway by inhibition of IgE by a peptide of the invention might result in a 10% reduction in food-specific IgE levels.

The compound useful in the present invention can be formulated as pharmaceutical compositions and administered to a mammalian host, such as a human patient or any animal in a variety of forms adapted to the chosen route of administration, *i.e.*, orally or parenterally, by intranasally by inhalation, intravenous, intramuscular, topical or subcutaneous routes.

The compound of the present invention can also be administered using gene therapy methods of delivery. See, *e.g.*, U.S. Patent No. 5,399,346, which is incorporated by reference

in its entirety. Using a gene therapy method of delivery, primary cells transfected with the gene for the compound of the present invention can additionally be transfected with tissue specific promoters to target specific organs, tissue, grafts, tumors, or cells.

Thus, the present compound may be systemically administered, *e.g.*, orally, in combination with a pharmaceutically acceptable vehicle such as an inert diluent or an assimilable edible carrier. They may be enclosed in hard or soft shell gelatin capsules, may be compressed into tablets, or may be incorporated directly with the food of the patient's diet. For oral therapeutic administration, the active compound may be combined with one or more excipients and used in the form of ingestible tablets, buccal tablets, troches, capsules, elixirs, suspensions, syrups, wafers, and the like. Such compositions and preparations should contain at least 0.1% of active compound. The percentage of the compositions and preparations may, of course, be varied and may conveniently be between about 2 to about 60% of the weight of a given unit dosage form. The amount of active compound in such therapeutically useful compositions is such that an effective dosage level will be obtained.

The tablets, troches, pills, capsules, and the like may also contain the following: binders such as gum tragacanth, acacia, corn starch or gelatin; excipients such as dicalcium phosphate; a disintegrating agent such as corn starch, potato starch, alginic acid and the like; a lubricant such as magnesium stearate; and a sweetening agent such as sucrose, fructose, lactose or aspartame or a flavoring agent such as peppermint, oil of wintergreen, or cherry flavoring may be added. When the unit dosage form is a capsule, it may contain, in addition to materials of the above type, a liquid carrier, such as a vegetable oil or a polyethylene glycol. Various other materials may be present as coatings or to otherwise modify the physical form of the solid unit dosage form. For instance, tablets, pills, or capsules may be coated with gelatin, wax, shellac or sugar and the like. A syrup or elixir may contain the active compound, sucrose or fructose as a sweetening agent, methyl and propylparabens as preservatives, a dye and flavoring such as cherry or orange flavor. Of course, any material used in preparing any unit dosage form should be pharmaceutically acceptable and substantially non-toxic in the amounts employed. In addition, the active compound may be incorporated into sustained-release preparations and devices.

The active compound may also be administered intravenously or intraperitoneally by infusion or injection. Solutions of the active compound or its salts can be prepared in water, optionally mixed with a nontoxic surfactant. Dispersions can also be prepared in glycerol, liquid polyethylene glycols, triacetin, and mixtures thereof and in oils. Under ordinary conditions of storage and use, these preparations contain a preservative to prevent the growth of microorganisms.

The pharmaceutical dosage forms suitable for injection or infusion can include sterile aqueous solutions or dispersions or sterile powders comprising the active ingredient which are adapted for the extemporaneous preparation of sterile injectable or infusible solutions or dispersions, optionally encapsulated in liposomes. In all cases, the ultimate dosage form must be sterile, fluid and stable under the conditions of manufacture and storage. The liquid carrier or vehicle can be a solvent or liquid dispersion medium comprising, for example, water, ethanol, a polyol (for example, glycerol, propylene glycol, liquid polyethylene glycols, and the like), vegetable oils, nontoxic glyceryl esters, and suitable mixtures thereof. The proper fluidity can be maintained, for example, by the formation of liposomes, by the maintenance of the required particle size in the case of dispersions or by the use of surfactants. The prevention of the action of microorganisms can be brought about by various antibacterial and antifungal agents, for example, parabens, chlorobutanol, phenol, sorbic acid, thimerosal, and the like. In many cases, it will be preferable to include isotonic agents, for example, sugars, buffers or sodium chloride. Prolonged absorption of the injectable compositions can be brought about by the use in the compositions of agents delaying absorption, for example, aluminum monostearate and gelatin.

Sterile injectable solutions are prepared by incorporating the active compound in the required amount in the appropriate solvent with various of the other ingredients enumerated above, as required, followed by filter sterilization. In the case of sterile powders for the preparation of sterile injectable solutions, the preferred methods of preparation are vacuum drying and the freeze drying techniques, which yield a powder of the active ingredient plus any additional desired ingredient present in the previously sterile-filtered solutions.

For topical administration, the present compound may be applied in pure form, i.e., when they are liquids. However, it will generally be desirable to administer them to the skin as

compositions or formulations, in combination with a dermatologically acceptable carrier, which may be a solid or a liquid.

Useful solid carriers include finely divided solids such as talc, clay, microcrystalline cellulose, silica, alumina and the like. Useful liquid carriers include water, hydroxyalkyls or glycols or water-alcohol/glycol blends, in which the present compound can be dissolved or dispersed at effective levels, optionally with the aid of non-toxic surfactants. Adjuvants such as fragrances and additional antimicrobial agents can be added to optimize the properties for a given use. The resultant liquid compositions can be applied from absorbent pads, used to impregnate bandages and other dressings, or sprayed onto the affected area using pump-type or aerosol sprayers.

Thickeners such as synthetic polymers, fatty acids, fatty acid salts and esters, fatty alcohols, modified celluloses or modified mineral materials can also be employed with liquid carriers to form spreadable pastes, gels, ointments, soaps, and the like, for application directly to the skin of the user.

Examples of useful dermatological compositions which can be used to deliver the compound to the skin are known to the art; for example, see Jacquet et al. (U.S. Pat. No. 4,608,392), Geria (U.S. Pat. No. 4,992,478), Smith et al. (U.S. Pat. No. 4,559,157) and Wortzman (U.S. Pat. No. 4,820,508).

Useful dosages of the compound can be determined by comparing their *in vitro* activity, and *in vivo* activity in animal models. Methods for the extrapolation of effective dosages in mice, and other animals, to humans are known to the art; for example, see U.S. Pat. No. 4,938,949.

Generally, the concentration of the compound(s) in a liquid composition, such as a lotion, will be from about 0.1-25 wt-%, preferably from about 0.5-10 wt-%. The concentration in a semi-solid or solid composition such as a gel or a powder will be about 0.1-5 wt-%, preferably about 0.5-2.5 wt-%.

The amount of the compound, or an active salt or derivative thereof, required for use in treatment will vary not only with the particular salt selected but also with the route of

administration, the nature of the condition being treated and the age and condition of the patient and will be ultimately at the discretion of the attendant physician or clinician. Also the dosage of the compound varies depending on the target cell, tumor, tissue, graft, or organ.

The desired dose may conveniently be presented in a single dose or as divided doses administered at appropriate intervals, for example, as two, three, four or more sub-doses per day. The sub-dose itself may be further divided, *e.g.*, into a number of discrete loosely spaced administrations; such as multiple inhalations from an insufflator or by application of a plurality of drops into the eye.

An administration regimen could include long-term, daily treatment. By "long-term" is meant at least two weeks and preferably, several weeks, months, or years of duration. Necessary modifications in this dosage range may be determined by one of ordinary skill in the art using only routine experimentation given the teachings herein. See Remington's Pharmaceutical Sciences (Martin, E.W., ed. 4), Mack Publishing Co., Easton, PA. The dosage can also be adjusted by the individual physician in the event of any complication.

FIGURES

Figure 1. Figure 1. Phage ELISA of PBL-derived VHH libraries against mouse serum albumin versus lymph node punctions

Figure 2. Sequences of VHHs raised against mouse serum albumin (SEQ ID NOs: 1 to 3), of bivalent constructs consisting of anti-mouse serum albumin VHH and anti-TNF-alpha VHH, wherein the underlined sequence is a linker (SEQ ID NOs: 4 to 6), of VHHs directed against IgE (SEQ ID NOs: 7 to 16), of VHHs directed against TNF-alpha (SEQ ID NOs: 17 to 29), of VHHs directed against vWF (SEQ ID NOs: 30 to 39), of bivalent constructs consisting of anti-mouse serum albumin VHH and anti-vWF VHH, wherein the underlined sequence is a linker (SEQ ID NOs: 40 to 42), of bivalent constructs consisting of anti-mouse serum albumin VHH and anti-IgE VHH, wherein the underlined sequence is a linker (SEQ ID NOs: 43 to 45), of VHHs directed against IFN-gamma (SEQ ID NOs: 46 to 62) and bivalent constructs consisting of anti-mouse serum albumin VHH and anti-IFN-gamma VHH (SEQ ID NOs: 63 to 65).

Figure 3. Results of experiments to test cross-reactivity according to Example 8 wherein 1= pig plasma, 2= human plasma, 3= rat plasma, 4= mouse plasma, 5= rabbit plasma, 6= hamster plasma, 7= baboon plasma.

Figure 4. Binding of purified VHH to human serum albumin as tested by ELISA.

Figure 5. Binding of purified VHH to mouse serum albumin as tested by ELISA.

Figure 6. Sandwich ELISA to verify functionality of both VHHs in the bispecific protein.

Figure 7. Phage ELISA for vWF, Idomain of gpIa/IIa, gpIb, collagen and casein (negative control) for VHH libraries of llama 002 or llama 004 PBL (first and second bleeding) versus lymph node punctions.

Figure 8: Western blot for expression of the A1 and A3 domain of vWF on the surface of *E.coli* as a fusion with OprI.

Figure 9: Results of sequencing A1 or A3 specific binders according to Example 6.

Figure 10. Binding of purified C37, A50 or A38 to vWF measured by ELISA.

Figure 11: The results of experiments to determine the inhibitory effect of VHH upon the binding of vWF to collagen as in Example 3.

Figure 12: The results of experiments to determine the inhibitory effect of VHH upon the binding of vWF to the platelet receptor gpIb as in Example 4.

Figure 13: Agarose gel of DNAse digest of vWF fragment (aa 1371 to 111 985) with decreasing concentrations of DNAse.

Figure 14. Binding in ELISA to vWF for C37 stored at -20°C as compared to C37 incubated at 37°C for 196 hours.

Figure 15. Residual activity for C37 stored at -20°C as compared to C37 incubated at 37°C for up to 194 hours. C37 stability is compared to stability of a scFv specific for B3 antigen and a stabilised form, dsFv (stabilised by 2 disulphide bonds).

EXAMPLES

The invention is illustrated by the following non-limiting examples.

Example 1: Immunisation of llamas

One llama was immunized with human serum albumin (HSA). The immunization scheme is summarized in Table 1.

Day of immunization	HSA Llama006
0	100 µg
7	100 µg
14	50 µg
21	50 µg
28	50 µg
35	50 µg

Table 1: Immunisation scheme according to Example 1

Example 2: Repertoire cloning

Different sources for RNA extraction were used:

- 150 ml blood, between 4 and 10 days after the last antigen injection
- lymph node biopsy 4 days after the last antigen injection

Peripheral blood lymphocytes (PBLs) were isolated by centrifugation on a density gradient (Ficoll-Paque Plus Amersham Biosciences). PBLs and lymph node were used to extract total RNA (Chomczynski and Sacchi 1987) followed by synthesis of cDNA using a hexanucleotide random primer. The repertoire was amplified using two hinge-specific primers: AACAGTTAAGCTTCCGCTTGCGGCCGCGGAGCTGGGGTCTTCGCTGTGGTGCG and AACAGTTAAGCTTCCGCTTGCGGCCGCTGGTTGTGGTTTTGGTGTCTTGGGTT and a framework 1 specific primer: GAGGTBCARCTGCAGGASTCYGG. Fragments were digested with PstI and NotI and cloned into a phagemid vector. The repertoire was transformed in TG1 electrocompetent cells and plated on LB agar plates containing 100 µg/ml ampicillin and 2% glucose. Colonies were screened for the presence of insert by PCR with vector specific primers.

Example 3: Rescue of the library, phage preparation

Libraries were grown at 37°C in 60 ml 2xTY medium containing 2% glucose, and 100 µg/ml ampicillin, until the OD600nm reached 0.5. 10^{12} M13KO7 phages were added and the mixture was incubated at 37°C for 2 x 30 minutes, first without shaking, then with shaking at 100 rpm. Cells were centrifuged for 10 minutes at 4500 rpm at room temperature. The bacterial pellet was resuspended in 300 ml of 2xTY medium containing 100 µg/ml ampicillin and 25 µg/ml kanamycin, and incubated overnight at 30°C with vigorously shaking at 250 rpm. The overnight cultures were centrifuged for 15 minutes at 10,000 rpm at 4°C. Phages were PEG precipitated (20% poly-ethylene-glycol and 1.5 M NaCl) and centrifuged for 30 minutes at 10.000 rpm. The pellet was resuspended in 20 ml PBS. Phages were again PEG precipitated and centrifuged for 30 minutes at 20,000 rpm and 4°C. The pellet was dissolved in 5 ml PBS. Phages were titrated by infection of TG1 cells at OD600nm= 0.5 and plating on LB agar plates containing 100 µg/ml ampicillin and 2% glucose. The number of transformants indicates the number of phages (= pfu). The phages were stored at -80°C with 15% glycerol.

Example 4: Phage ELISA

A microtiter plate (Maxisorp) was coated overnight at 4°C with PBS-1% casein or with 5 µg/ml HSA or MSA. The plate was washed 3 times with PBS-Tween (0.05% Tween20) and blocked for 2 hours at room temperature with 200 µl PBS-1% casein. The plate was washed five times with PBS-Tween. Phages were prepared as described above and applied to the wells in consecutive twofold dilutions. Plates were washed five times with PBS-Tween. Bound phage were detected with a mouse monoclonal antibody anti-M13 conjugated with horse radish peroxidase (HRP) diluted 1/2000 in PBS. The plates were washed five times with PBS-Tween. Staining was performed with ABTS/H_2O_2 and signals were measured after 30 minutes at 405 nm (see Figure 1 for HSA).

Example 5: Selection: first and second round of biopanning

A well in a microtiterplate was coated with 10 µg/ml mouse serum albumin (MSA), or with PBS containing 1% casein. After overnight incubation at 4°C, the wells were blocked with PBS containing 1% casein, for 3 hours at RT. 200 µl phages of the three libraries were pooled and 100 µl of this pool was added to the wells. After 2 hours incubation at RT, the wells were washed 10x with PBS-Tween and 10x with PBS. Bound phages were eluted with 100 µl 0.2 M glycin buffer pH= 2.4. Elutions were performed for 20 minutes at room

temperature. Eluted phages were allowed to infect exponentially growing *E. coli* TG1 cells, and were then plated on LB agar plates containing 100 µg/ml ampicillin and 2% glucose. A second round was performed with the same conditions as described above. Results are summarized in Table 2.

	First round	Second round
Pfu mouse serum albumin	2.5×10^7	2.5×10^7
Pfu casein	5×10^3	2.5×10^3
Enrichment	5,000	10,000

Table 2: Results of first and second round selection of anti-MSA VHH according to Example 5

Example 6: Screening of individual clones after biopanning

ELISA: binding to human serum albumin (HSA) and mouse serum albumin (MSA)

A microtiter plate was coated with 5 µg/ml HSA, with 5 µg/ml mouse serum albumin (MSA) or with PBS-1% casein, overnight at 4°C. Plates were blocked for two hours at room temperature with 300 µl 1% casein in PBS. The plates were washed three times with PBS-Tween. Periplasmic fraction was prepared for 23 individual clones after the first and second round of selection, and allowed to bind to the wells of the microtiter plate. Plates were washed six times with PBS-Tween, after which binding of VHH was detected by incubation with mouse anti-Histidine monoclonal antibody Serotec MCA 1396 (1/1000 dilution) in PBS for 1 hour at RT followed by anti-mouse-alkaline phosphatase conjugate 1/2000 in PBS, also for 1 hour at RT. Staining was performed with the substrate PNPP (p-nitrophenyl-phosphate, 2 mg/ml in 1 M diethanolamine, 1 mM Mg_2SO_4, pH 9.8) and the signals were measured after 30 minutes at 405 nm. Results are summarized in Table 3

	First round	Second round
ELISA mouse serum albumin	1/16	15/16
ELISA human serum albumin	1/16	15/16
ELISA casein	0/16	0/16

Table 3. Results of biopanning according to Example 6.

Example 7: HinfI pattern and sequencing

A PCR was performed on 16 colonies after the second round of panning, with a set of primers binding to a sequence in the vector. The PCR product was digested with the restriction enzyme HinfI and loaded on a agarose gel. 3 clones were selected with a different HinfI-pattern for further evaluation. Those clones were sequenced, and results are summarized in Figure 2 (MSA21, MSA24, MSA210).

Example 8: test cross-reactivity with albumin of different species

A SDS-PAGE was run for plasma (1/10 dilution) from different species (baboon, pig, hamster, human, rat. mouse and rabbit) and blotted on a nitrocellulose membrane. Phages were prepared for clones MSA 21. MSA 24, MSA 210 and a irrelevant VHH as described in example 3. Phages were allowed to bind to the nitrocellulose blotted serum albumins and unbound phages were washed away. Binding was detected with a anti-M13 polyclonal antibody coupled to HRP. DAP was used as a substrate for detection. Results are shown in Figure 3.

From these results we can conclude that all 4 binders are cross-reactive between pig, human, mouse and hamster serum albumin. MSA 21 is also cross-reactive with rabbit serum albumin.

Example 9: Expression and purification

Plasmid was prepared for the binders and was transformed into WK6 electrocompetent cells. A single colony was used to start an overnight culture in LB containing 2% glucose and 100 µg/ml ampicillin. This overnight culture was diluted 100-fold in 300 µl TB medium containing 100 µg/ml ampicillin, and incubated at 37°C until OD600nm= 0.5. 1 mM IPTG was added and the culture was incubated for 3 more hours at 37°C or overnight at 28°C.

Cultures were centrifuged for 20 minutes at 10.000 rpm at 4°C. The pellet was froozen overnight or for 1 hour at -20°C. Next, the pellet was thawed at room temperature for 40 minutes, re-suspended in 20 ml PBS and shaken on ice for 1 hour. Periplasmic fraction was isolated by centrifugation for 20 minutes at 4°C at 20.000 rpm. The supernatant containing the VHH was loaded on Ni-NTA and purified to homogeneity.

Example 10: ELISA on purified VHH

An ELISA was performed with the purified VHH as described in Example 6. VHH were applied in dilutions starting at a concentration of 2 μM. VHH were tested for binding to MSA, HSA and PBS-1% casein. No binding was observed for PBS-1% casein. Results for HSA and MSA are summarized in Figures 4 and 5.

Example 11: Making bispecific constructs

Bispecific constructs were made as described in patent application number WO 96/34103. The middle hinge of llama was used as a linker between the VHHs. A VHH against TNF-alpha was cloned at the COOH terminal of MSA specific VHHs. Plasmid was prepared and was transformed into WK6 electrocompetent cells. A single colony was used to start an overnight culture in LB containing 2% glucose and 100 μg/ml ampicillin. This overnight culture was diluted 100-fold in 300 μl TB medium containing 100 mg/ml ampicillin, and incubated at 37°C until OD600nm= 0.5. 1 mM IPTG was added and the culture was incubated for 3 more hours at 37°C.

Cultures were centrifuged for 20 minutes at 10.000 rpm at 4°C. The pellet was froozen overnight at -20C. The next morning, the pellet was thawed in the cold room for 40 minutes, re-suspended in 20 ml PBS and shaken on ice for 1 hour. Periplasmic fraction was isolated by centrifugation for 20 minutes at 4°C at 10.000 rpm. The supernatant was loaded on Ni-NTA and purified to homogeneity. Sequences are shown in Figure 2 (MSA 21/TNF3E, MSA 24/TNF3E, MSA 210/TNF3E). The hinge is underlined.

Example 12: Testing bispecific construct in sandwich ELISA

A microtiter plate was coated with 5 μg/ml MSA overnight at 4°C. Plates were blocked for two hours at room temperature with 300 μl 1% casein in PBS. The plates were washed three times with PBS-Tween. Purified protein for the bispecific constructs was allowed to bind to the wells of the microtiterplate at a concentration of 2 μg/ml. Plates were washed six times with PBS-Tween, Biotinilated TNF was added at a concentration of 10 μg/ml and diluted 3 fold, and allowed to bind for 2 hours at room temperature. Binding was detected by incubation with mouse extravidin alkaline phosphatase conjugate (Sigma) 1/2000 in PBS, for 1 hour at RT. Staining was performed with the substrate PNPP (p-nitrophenyl-phosphate, 2 mg/ml in

1M diethanolamine, 1mM Mg_2SO_4, pH9.8) and the signals were measured after 30 minutes at 405 nm. Results are shown in Figure 6 and indicate that the bispecific construct can bind both antigens simultaneously.

Example 13: Pharmacokinetics in mice

Monovalent and bispecific protein was administered as a bolus in mice at 0.5 mg/kg. At different time points after administration, blood samples were collected and the presence of monovalent or bispecific protein was tested in ELISA as described in Example 8.

Example 14: VHH directed against IgE

Two llama's were immunized with Human IgE, Scripps laboratories, Cat nr. I0224. The following immunization schemes were used according to Table 4.

Day	Llama 2	Llama 4
0	100 µg	100 µg
7	100 µg	
14	50 µg	
21	50 µg	100 µg
28	50 µg	
35	50 µg	
42		50 µg
70		50 µg

Table 4: Immunisation scheme

Different sources for RNA extraction were used:

- 150 ml immune blood, between 4 and 10 days after the last antigen injection
- lymph node biopsy 4 days after the last antigen injection

Peripheral blood lymphocytes (PBLs) were isolated by centrifugation on a density gradient (Ficoll-Paque Plus Amersham Biosciences). PBLs and lymph node were used to extract total RNA (Chomczynski and Sacchi 1987) followed by synthesis of cDNA using a hexanucleotide random primer. The repertoire was amplified using two hinge-specific primers:

AACAGTTAAGCTTCCGCTTGCGGCCGCGGAGCTGGGGTCTTCGCTGTGGTGCG and AACAGTTAAGCTTCCGCTTGCGGCCGCTGGTTGTGGTTTTGGTGTCTTGGGTT and a framework 1 specific primer:

GAGGTBCARCTGCAGGASTCYGG.

Fragments were digested with PstI and NotI and cloned into a phagemid vector. The repertoire was transformed in TG1 electrocompetent cells and plated on LB agar plates containing 100 □g/ml ampicillin and 2% glucose. Colonies were screened for the presence of insert by PCR with vector specific primers. Results are summarized in Table 5:

	#days after last injection	Source RNA	Size of the library	% insert
Llama002	4	Lymph	1.3×10^7	89
	4	PBL	1.9×10^7	95
	10	PBL	1.1×10^9	70
Llama004	4	PBL	1.7×10^8	96
	4	Lymph	4.9×10^7	>95
	10	PBL	2.2×10^8	>95

Table 5. Results of screening

Selections were done using chimaeric IgE instead of human IgE, used for immunization, in order to select for VHH molecules directed against the constant region of IgE. The region interacting with the Fcεreceptor is located in the constant part of IgE, more in particular in the region covered by Cε2-Cε3.

A first selection was performed using the pool of PBL day4, PBL day10 and lymph node day4 libraries for each of the two llama's. Chimaeric IgE was solid phase coated at 5 µg/ml and 0.5 µg/ ml and specific phages were eluted using 0.1 M glycine pH = 2.5.
The results obtained are shown in Table 6.

	5 µg/ ml	0.5 µg/ ml	0 µg/ ml (blank)
Llama 2 (pool PBL day4, PBLday10, lymph node day4)	$1.4\ 10^6$	$2.7\ 10^5$	$1.5\ 10^4$
Enrichment compared to blank	*400 x*	*18 x*	
Llama 4 (pool PBL day4, PBLday10, lymph node day4)	$3.3\ 10^6$	$4.5\ 10^5$	$7.2\ 10^4$
Enrichment compared to blank	*140 x*	*6.25 x*	

Table 6. Results of first selection

A second selection was performed using the rescued phages from the first selection using 5 µg/ ml. Chimaeric IgE was solid phase coated at 1 µg/ml and specific phages were eluted using buffy coat cells or lysozyme for 1 hr. Buffy coat cells contain cells expressing the Fcεreceptor, while lysozyme is an irrelevant protein and serves as a control. The results obtained are shown in Table 7:

	1 µg/ ml	1 µg/ ml	0 µg/ ml	0 µg/ ml
	Elution buffy coat cells	Elution Lysozyme	Elution buffy coat cells	Elution Lysozyme
Llama 2 (selection 5 µg/ ml IgE: 400 x enrichment)	$1.2\ 10^6$	$1.2\ 10^6$	$6\ 10^3$	$3\ 10^3$
Enrichment compared to lysozyme elution	No enrichment			2x
Llama 4 (selection 5 µg/ ml IgE: 140 x enrichment)	$1.3\ 10^6$	$2\ 10^7$	$3\ 10^3$	$3\ 10^3$
Enrichment compared to lysozyme elution	6.5 x		No enrichment	

Table 7. Results of second selection

Another second round selection was performed using neutravidine coated tubes and 2 nM biotinylated IgE. Specific phages were eluted using buffy coat cells or lysozyme for 1 hr. Buffy

coat cells contain cells expressing the Fcεreceptor, while lysozyme is an irrelevant protein and serves as a control. The results obtained are shown in Table 8:

	2 nM IgE	2 nM IgE	0 nM IgE	0 nM IgE
	Elution buffy coat cells	Elution Lysozyme	Elution buffy coat cells	Elution Lysozyme
Llama 2 (selection 5 µg/ ml IgE: 400 x enrichment)	$1.5\ 10^{8}$	$1.5\ 10^{7}$	$3\ 10^{5}$	$3\ 10^{3}$
Enrichment compared to lysozyme elution	10 x			
Llama 4 (selection 5 µg/ ml IgE: 140 x enrichment)	$3.3\ 10^{7}$	$2.2\ 10^{7}$	$3\ 10^{3}$	$6\ 10^{3}$
Enrichment compared to lysozyme elution	1.5 x			

Table 8. Results of additional second round selection

Individual clones obtained from the first round of selection were screened in an ELISA using solid phase coated human IgE or chimaeric IgE. The number of clones that score positive for binding to both human IgE and chimeric IgE versus the number of clones tested in ELISA are summarised in Table 9:

	Selection with 5 µg/ml	Selection with 0.5 µg/ml
Llama 002	39/47	21/47
Llama 004	45/47	46/47

Table 9. Number of clones scoring positive for binding to both human IgE and chimeric IgE versus the number of clones tested in the ELISA

Clones were picked which were positive for human and chimaeric IgE binding, amplified by PCR and digested with HinfI. HinfI profiles were determined on agarose gel and representative clones for different profiles were sequenced. The sequences obtained are shown in Figure 2.

Example 15: VHH directed against vWF - Immunization of llamas

2 llamas were immunized with a cocktail of vWF, platelet receptor gpIb, platelet receptor gpIa/IIa (α_2I-domain) and collagen. The immunization schemes are summarized in Tables 10 and 11.

Llama002 Day of immunization	vWF	rgpIb	gpIa/Ia α_2I-domain	Collagen
0	100 µg	40 µg	40 µg	100 µg
7	100 µg	40 µg	40 µg	100 µg
14	50 µg	20 µg	20 µg	50 µg
21	50 µg	20 µg	20 µg	50 µg
28	50 µg	20 µg	20 µg	50 µg
35	50 µg	20 µg	20 µg	50 µg

Table 10. Immunisation scheme according to Example 15

Llama004 Day of immunization	vWF	rgpIb	gpIa/Ia α_2I-domain	Collagen
0	100 µg	40 µg	40 µg	100 µg
21	100 µg	40 µg	40 µg	100 µg
42	50 µg	20 µg	20 µg	50 µg
70	50 µg	20 µg	20 µg	50 µg

Table 11. Immunisation scheme according to Example 15

Example 16: VHH directed against vWF - Repertoire cloning

Different sources for RNA extraction were used:

- 150 ml immune blood, between 4 and 10 days after the last antigen injection
- lymph node biopsy 4 days after the last antigen injection

Peripheral blood lymphocytes (PBLs) were isolated by centrifugation on a density gradient (Ficoll-Paque Plus Amersham Biosciences). PBLs and lymph node were used to extract total RNA (Chomczynski and Sacchi 1987) followed by synthesis of cDNA using a hexanucleotide random primer. The repertoire was amplified using two hinge-specific primers: AACAGTTAAGCTTCCGCTTGCGGCCGCGGAGCTGGGGTCTTCGCTGTGGTGCG and AACAGTTAAGCTTCCGCTTGCGGCCGCTGGTTGTGGTTTTGGTGTCTTGGGTT and a framework 1 specific primer: GAGGTBCARCTGCAGGASTCYGG. Fragments were digested with PstI and NotI and cloned into a phagemid vector. The repertoire was transformed in TG1 electrocompetent cells and plated on LB agar plates containing 100 µg/ml ampicillin and 2%

glucose. Colonies were screened for the presence of insert by PCR with vector specific primers. Results are summarised in Table 12.

	#days after last injection	Source RNA	Size of the library	% insert
Llama002	4	Lymph	1.3×10^7	89
	7	PBL	1.9×10^7	95
	10	PBL	1.1×10^8	70
Llama004	4	PBL	1.7×10^8	96
	4	Lymph	4.9×10^7	>95

Table 12. Results of screening according to Example 16.

Example 17: VHH directed against vWF - Rescue of the library, phage preparation

Libraries were grown at 37°C in 60 ml 2xTY medium containing 2% glucose, and 100 µg/ml ampicillin, until the OD600nm reached 0.5. M13KO7 phages (1012) were added and the mixture was incubated at 37°C for 2 x 30 minutes, first without shaking, then with shaking at 100 rpm. Cells were centrifuged for 10 minutes at 4500 rpm at room temperature. The bacterial pellet was resuspended in 300 ml of 2xTY medium containing 100 µg/ml ampicillin and 25 µg/ml kanamycin, and incubated overnight at 30°C with vigorously shaking at 250 rpm. The overnight cultures were centrifuged for 15 minutes at 10.000 rpm at 4°C. Phages were PEG precipitated (20% poly-ethylene-glycol and 1.5 M NaCl) and centrifuged for 30 minutes at 10.000 rpm. The pellet was resuspended in 20 ml PBS. Phages were again PEG precipitated and centrifuged for 30 minutes at 20,000 rpm and 4°C. The pellet was dissolved in 5 ml PBS. Phages were titrated by infection of TG1 cells at OD600nm= 0.5 and plating on LB agar plates containing 100 µg/ml ampicillin and 2% glucose. The number of transformants indicates the number of phages (= pfu). The phages were stored at –80°C with 15% glycerol.

Example 18: VHH directed against vWF - Phage ELISA

A microtiter plate (Maxisorp) was coated overnight at 4°C with PBS-1% casein, 2 µg/ml vWF, 10 µg/ml gpIb or 25 µg/ml gpIa/IIa α_2I-domain in carbonate buffer containing 5 mM $MgCl_2$, or coated overnight at 4°C with 1% casein and 25µg/ml collagen in PBS. The plate was washed 3 times with PBS-Tween (0.05% Tween20) and blocked for 2 hours at room temperature with 200 µl PBS-1% casein. The plate was washed five times with PBS-Tween. Phages were prepared as described above and applied to the wells in duplo dilutions. Plates were washed five times with PBS-Tween. Binding phages were detected with a mouse anti-M13 conjugated

with HRP diluted 1/2000 in PBS. The plates were washed five times with PBS-Tween. Staining was performed with ABTS/H2O2 and signals were measured after 30 minutes at 405 nm. The results are presented in Figure 7. It is clear from the results that specific VHH are present in all libraries for all the different antigens.

Example 19: VHH directed against vWF - Selection

Immunotubes were coated with 2 µg/ml vWF or with PBS containing 1% casein. After overnight incubation at 4°C, the tubes were blocked with PBS containing 1% casein, for 3 hours at RT. 200 µl phages of the three libraries of llama002 were pooled and added to the immunotubes with a final volume of 2 ml in PBS. After 2 hours incubation at RT, the immunotubes were washed 10x with PBS-Tween and 10x with PBS. Bound phages were eluted with 2 ml 0.2 M glycin buffer pH= 2.4 or with 2 ml of a 100 µg/ml collagen solution. Elutions were performed for 20 minutes or 1 hour resp. at room temperature. Alternatively, 200 µl phages of llama004 were added to the immunotubes and bound phages were eluted with 100 µg/ml collagen solution. Eluted phages were allowed to infect exponentially growing TG1 cells, and were then plated on LB agar plates containing 100 µg/ml ampicillin and 2% glucose. The results from the panning are presented in Table 13.

llama	Source RNA	Elution conditions	Pfu vWF	Pfu casein	Enrichment
002	Pool of the 3 libraries	0.2 M glycin, pH 2.4	1.5×10^7	1×10^4	1.500
002	Pool of the 3 libraries	100 µg/ml collagen	4.5×10^6	3.2×10^4	140
004	PBL day 4	100 µg/ml collagen	1.8×10^5	250	720
004	Lymph day 4	100 µg/ml collagen	3.6×10^5	250	1.440

Table 13: Results of panning according to Example 19

Example 20: VHH directed against vWF - Screening

Cloning A1 and A3 domain of vWF in pBAD-OprI-ss

The pBAD-OprI-strep-spec vector was used to display the VWF A1 and A3 domains as a fusion with OprI on the surface of UT5600 *E.coli* cells (F- ara-14 leuB6 azi-6 lacY1 proC14 tsx-67 entA403 trpE38 rfbD1 rpsL109 xyl-5 mtl-1 thi1 DompT fepC266) (Cote-Sierra et al, 1998, *Gene*, 221: 25-34). The gene coding for the A1 domain of vWF (219aa) was amplified by PCR using the A1for and A1back PCR primers. The gene coding for the A3 domain of vWF (201aa) was amplified by PCR using the A3for and A3back PCR primers.

A1for: CCG GTG AGC CCC ACC ACT CTA AGC TTG GAG GAC ATC TCG GAA CCG

A1back: CCC CAG GGT CGA AAC CCT CTA GAG CCC CGG GCC CAC AGT GAC

A3for: CTG GTG CTG CAG AGG TGA AGC TTC GGA GAG GGG CTG CAG ATC

A3back: ATC CAT GCA AAT CCT CTA GAA TCC AGA GCA CAG TTT GTG GAG

Fragment and vector were digested with HindIII and XbaI, ligated and transformed in UT5600 (= pBAD-vWFA1/pBAD-vWFA3). Transformed cells were plated on LB agar plates containing 20 µg/ml streptomycin, 50 µg/ml spectinomycin.

The pBAD-vWFA1/pBAD-vWFA3 plasmids were transformed in UT5600 F- cells and plated on LB agar plates with 20 µg/ml streptomycin, 50 µg/ml spectinomycin. A single colony was used to inoculate LB medium with 20 µg/ml streptomycin, 50 µg/ml spectinomycin. Cells were grown overnight at 37°C at 200 rpm. The next day, cells were induced with 0.2% arabinose and incubated for 1 more hour at 37°C at 150 rpm. Total cell lysates were boiled in reducing sample buffer, loaded on a 12% SDS-PAGE and transferred to nitrocellulose for Western blotting. Transferred proteins were detected using a monoclonal anti-OprI antibody (SH2.2) (Cote-Sierra et al, 1998, *Gene*, 221: 25-34). An anti-mouse IgG conjugated with alkaline phosphatase was applied (Sigma), and the blots were developed with BCIP/NBT (figure 8).

ELISA: binding of phage expressing VHH to *E.coli* cells expressing the A1 or A3 domain on their surface.

The pBAD-vWFA1/pBAD-vWFA3 plasmids were transformed in UT5600 F- cells and plated on LB agar plates with 20 µg/ml streptomycin, 50 µg/ml spectinomycin. A single colony was used to inoculate LB medium with 20 µg/ml streptomycin, 50 µg/ml spectinomycin. Cells were grown overnight at 37°C at 200 rpm. The next day, cells were induced with 0.2% arabinose and incubated for 1 more hour at 37°C at 150 rpm. A microtiter plate was coated overnight at 4°C with the monoclonal anti-OprI antibody (SH2.2) diluted 1/1000 in PBS. After induction, total cells were allowed to bind to the plate for 1 hour at room temperature. The plates were washed five times with PBS-Tween. Phage preparations of single colonies were allowed to bind for two hours at room temperature. The plates were washed five times with PBS-Tween. An anti-M13 HRP conjugate was used for detection of phage binding to *E. coli* cells expressing the A1 or A3 domain on their surface. The plates were washed five times with PBS-Tween. Staining was performed with ABTS/H2O2 and signals were measured after 30

minutes at 405 nm. The number of positive clones for each phage preparation is presented in Table 14. A1 or A3 specific binders were sequenced. Sequenced results revealed the presence of 5 different binders for the A1 domain of vWF isolated from llama 002, 1 specifc binder for the A3 domain of the vWF from llama 002 (C37) and from llama 004 (T76). The results of sequencing some A1 or A3 binders is given in Figure 9.

ELISA: binding to vWF

A microtiter plate was coated with 2 µg/ml vWF, overnight at 4°C. Plates were blocked for two hours at room temperature with 300 µl 1% casein in PBS. The plates were washed three times with PBS-Tween. Dilution series (150 µg/ml to 0.28 ng/ml, dilution factor three) of all purified samples were incubated for 2 hours at RT. Plates were washed six times with PBS-Tween, after which binding of VHH was detected by incubation with mouse anti-Histidine mAB 1/1000 in PBS for 1 hour at RT followed by anti-mouse-alkaline phosphatase conjugate 1/2000 in PBS, also for 1 hour at RT. Staining was performed with the substrate PNPP (p-nitrophenyl-phosphate, 2 mg/ml in 1M diethanolamine, 1mM Mg_2SO_4, pH9.8) and the signals were measured after 30 minutes at 405 nm. The number of positive clones for each selection is presented in Table 14, and the binding as a function of concentration of purified C37, A50 and A38 is indicated in Figure 10.

llama	Elution Buffer	No. clones +ve for vWF / No. tested	No. clones +ve for A1 / No. tested	No. clones +ve for A3 / No. tested
002	0.2M glycine, pH 2.4	344/380	5/570	2/380
002	100 µg/ml collagen	356/380	0/380	3/380
004	100 µg/ml collagen	-	0/96	1/96
004	100 µg/ml collagen	-	0/96	7/96

Table 14: Number of positive clones for each phage in the ELISAs of Example 20

Example 21: VHH directed against vWF - Functional characterization of A1 or A3 binders: Inhibition of binding of vWF to collagen by VHH

A microtiter plate was coated overnight at 4°C with collagen type VIII at 25 µg/ml in PBS. The plate was washed five times with PBS-tween and blocked for 2 hours at room temperature

with PBS containing 1% casein. The plate was washed five times with PBS-tween. 60 µl plasma containing vWF (1/20 dilution in PBS-0.1% casein (plasma is incubated at 37°C for 15 minutes)) was mixed with 60 µl periplasmic extract containing a VHH antibody for testing and incubated for 30 minutes at room temperature. 60 µl of this mixture was applied to a well coated with collagen, and incubated for 90 minutes at room temperature. The plate was washed five times with PBS-tween. An anti-vWF-HRP monoclonal antibody was diluted 3,000-fold in PBS and incubated for 1 hour. The plate was washed five times with PBS-tween and vWF-binding was detected with ABTS/H2O2. Signals were measured after 30 minutes at 405 nm. The results presented in Figure 11, demonstrates that C37 specifically competes with collagen for binding to vWF. A50 here is a negative control.

Example 22: VHH directed against vWF - Functional characterization of A1 or A3 binders: Inhibition of binding of vWF to the platelet receptor gpIb

A microtiter plate was coated overnight at 4°C with an antibody specific for platelet receptor gpIb at 5µg/ml in PBS. The plate was washed five times with PBS-Tween, and blocked with 300 µl PBS-1% casein for 2 hours at room temperature. The plate was washed 3 times with PBS-Tween. Platelet receptor gpIb (gpIb) was applied to the wells of the microtiter plate at a concentration of 1 µg/ml and allowed to bind for 2 hours at room temperature. The plate was washed five times with PBS-Tween. Plasma containing vWF was pre-incubated at a dilution of 1/128 at 37°C for 5 minutes. Risto was added at a final concentration of 760 µg/ml and VHH. This mixture was incubated for 30 minutes at room temperature. 100 µl of this mixture was then applied to a microtiter plate well and incubated for 90 minutes at room temperature. The plate was washed five times with PBS-Tween. A anti-vWF-HRP monoclonal antibody was diluted 3.000-fold in PBS and incubated for 1 hour. The plate was washed five times with PBS-tween and vWF-binding was detected with ABTS/H2O2. Signals were measured after 30 minutes at 405 nm. The results presented in Figure 12, demonstrate that A38 and A50 specifically competes with platelet receptor gpIb for binding to vWF.

Example 23: VHH directed against vWF - Epitope mapping

A library of fragments of vWF was prepared in pBAD-OprI-ss. Therefore, the gene coding from aa 1371 to aa 1985 (A1-A2-A3 domain of vWF) was amplified by PCR and digested with decreasing concentrations of DNAse (see Figure 13). Fragments were amplified with DNAse

primer (AAGCTT(C)17) and XbaI primer (TCTAGA(C)17). Fragment and vector were digested with HindIII and XbaI, ligated and transformed in UT5600 *E.coli* cells. Transformed cells were plated on LB agar plates containing 20 µg/ml streptomycin, 50 µg/ml spectinomycin. The size of the libraries varies between 4 to20 x 104 cfu. Single colonies were grown in 96-well plates and captured on SH2.2-coated ELISA plates. Phages were prepared for a A3 specific VHH (C37) and binding to the OprI fusion expressed DNAse digested vWF fragments was analyzed as described above for A1 and A3. The results indicate the epitope mapped by C37.

Example 24: VHH directed against vWF - Expression and purification of VHH

Plasmid was prepared for specific A1 or A3 binders and transformed into WK6 electrocompetent cells. A single colony was used to start an overnight culture in LB containing 2% glucose and 100 µg/ml ampicillin. This overnight culture was diluted 100-fold in 300 µl TB medium containing 100 mg/ml ampicillin, and incubated at 37°C until OD600nm= 0.5. 1 mM IPTG was added and the culture was incubated overnight at 30°C.

Overnight cultures were centrifuged for 20 minutes at 10.000 rpm at 4°C. The pellet was resuspended in 10 ml TES (0.2 M Tris-HCl, 0.5 mM EDTA, 0.5 M sucrose, pH= 8.0) and incubated on ice for 20 minutes. 15 ml TES/4 was added and the mixture was incubated for 30 minutes at 4°C. Periplasmic fraction was isolated by centrifugation for 20 minutes at 4°C at 10.000 rpm. The supernatant containing the VHH was loaded on Ni-NTA and purified to homogeneity. The yield of VHH was calculated according to the extinction coefficient. The results are presented in Table 15.

VHH code name	Epitope	Extinction coefficient	Yield (mg pure VHH / liter culture)
A50	A1	1.275	5.7
A38	A1	1.671	1.4
C37	A3	1.907	3.3

Table 15: Yield of VHH according to Example 24

Example 25: Stability of VHH at 37°C

VHH C37 was incubated at 37°C and binding activity for vWF was measured at different time points by ELISA as described above. Results were compared to VHH stored at -20°C and are presented in Figure 14.

VHH C37 was incubated at 37°C for up to 194 hours and its activity was measured at certain time points. The results are presented in Figure 15. Shown for comparison are the activities of a scFv against B3 antigen (Reiter et al, Protein Engineering, 1994, 7: 697-704), and said scFv modified by the introduction of a disulphide bond between framework residues 44 and 105 to enhance its stability (dsFv), after incubation at 37°C. The results indicate that C37 lost no activity, even after 194 hours at 37°C, while dsFv lost 40% of its activity after 60 hours incubation at 37°C.

Example 26: Experiments to test the polypeptides of the present invention in animal models

The effects of polypeptides represented by SEQ ID NOs: 40 to 42, homologous sequences thereof and functional portions thereof upon platelet-mediated aggregation are being tested using an animal model. Polypeptides are being tested using the methods described in PCT application number WO 02/051351 A2.

Claims

1. A polypeptide construct comprising:
- one or more single domain antibodies each directed against one or more therapeutic and/or diagnostic targets, and
- one or more single domain antibodies each directed against one or more serum proteins.

2. A polypeptide construct according to claim 1 directed against a single target wherein said anti-target single domain antibodies do not share the same sequence.

3. A polypeptide construct according to claims 1 and 2 directed against a single serum protein wherein said anti-serum protein single domain antibodies do not share the same sequence.

4. A polypeptide construct according to any of claims 1 to 3 wherein said single domain antibodies are *Camelidae* VHHs antibodies.

5. A polypeptide construct according to any of claims 1 to 4 wherein said one or more serum protein are any of serum albumin, serum immunoglobulins, thyroxine-binding protein, transferring, or fibrinogen or a fragment thereof.

6. A polypeptide construct according to claim 1 to 5 wherein said one or more single domain anti-serum protein antibodies correspond to one or more sequences represented by any of SEQ ID NOs: 1 to 3.

7. A polypeptide construct according to any of claims 1 to 6 directed against a single target, wherein said target is Tumour Necrosis Factor-alpha.

8. A polypeptide construct according to claim 7 wherein said one or more single domain anti-target antibodies correspond to one or more sequences represented by any of SEQ ID NOs: 17 to 29.

9. A polypeptide construct according to claim 7 corresponding to the sequence represented by any of SEQ ID NO: 4 to 6.

10. A polypeptide construct according to any of claims 7 to 10, wherein said polypeptide construct is a homologous sequence of said polypeptide construct, a functional portion thereof, of an homologous sequence of a functional portion thereof.

11. A nucleic acid encoding a polypeptide construct according to any of claims 7 to 10.

12. A polypeptide construct according to any of claims 7 to 10, or a nucleic acid according to claim 11 for use in the treatment, prevention and/or alleviation of disorders relating to inflammatory processes.

13. Use of a polypeptide construct according to any of claims 7 to 10, or a nucleic acid according to claim 11 for the preparation of a medicament for the treatment, prevention and/or alleviation of disorders relating to inflammatory processes.

14. A polypeptide construct or nucleic acid according to claim 12 or a use of a polypeptide construct according to claim 13 wherein said disorders are any of rheumatoid arthritis, Crohn's disease, ulcerative colitis and multiple sclerosis.

15. A polypeptide construct or nucleic acid according to claims 12 and 14 or a use of a polypeptide construct according to claim 13 and 14 wherein said polypeptide construct is administered intravenously, orally, sublingually, topically, nasally, vaginally, rectally or by inhalation.

16. A polypeptide construct according to any of claims 1 to 6 directed against a single target wherein said target is vWF

17. A polypeptide construct according to claims 1 to 6 directed against a single target wherein said target is collagen.

18. A polypeptide construct according to claim 16 wherein said one or more anti-target single domain antibodies correspond to one or more sequences represented by any of SEQ ID NOs: 30 to 39.

19. A polypeptide construct according to claim 16 corresponding to the sequence represented by any of SEQ ID NOs: 40 to 42.

20. A polypeptide construct according to any of claims 16 to 19, wherein said polypeptide construct is a homologous sequence of said polypeptide construct, a functional portion thereof, of an homologous sequence of a functional portion thereof.

21. A nucleic acid encoding a polypeptide construct according to any of claims 16 to 20.

22. A polypeptide construct according to any of claims 16 to 20, or a nucleic acid according to claim 16 for use in the treatment, prevention and/or alleviation of disorders or conditions relating to platelet-mediated aggregation or dysfunction thereof.

23. Use of a polypeptide construct according to any of claims 16 to 20, or a nucleic acid according to claim 16 for the preparation of a medicament for the treatment, prevention and/or alleviation of disorders or conditions relating to platelet-mediated aggregation or dysfunction thereof.

24. A polypeptide construct or nucleic acid according to claim 22 or a use of a polypeptide construct or nucleic acid according to claim 23 wherein said disorders are any of cerebral ischemic attack, unstable angina pectoris, cerebral infarction, myocardial infarction, peripheral arterial occlusive disease, restenosis, and said conditions are those arising from coronary by-pass graft, or coronary artery valve replacement and coronary interventions such angioplasty, stenting, or atherectomy.

25. A polypeptide construct or nucleic acid according to claims 22 and 24 or a use of a polypeptide construct according to claim 23 and 24 wherein said polypeptide construct is administered intravenously, orally, sublingually, topically, nasally, vaginally, rectally or by inhalation.

26. A polypeptide construct according to any of claims 1 to 6 directed against a single target wherein said target is IgE.

27. A polypeptide construct according to claim 26 wherein said one or more anti-target single domain antibodies correspond to one or more sequences represented by any of SEQ ID NOs: 7 to 16.

28. A polypeptide construct according to claim 26 corresponding to the sequence represented by any of SEQ ID NOs: 43 to 45.

29. A polypeptide construct according to any of claims 26 to 28, wherein said polypeptide construct is a homologous sequence of said polypeptide construct, a functional portion thereof, of an homologous sequence of a functional portion thereof.

30. A nucleic acid encoding a polypeptide construct according to any of claims 26 to 29.

31. A polypeptide construct according to any of claims 26 to 29, or a nucleic acid according to claim 30 for use in the treatment, prevention and/or alleviation of disorders or conditions relating to allergic reactions.

32. Use of a polypeptide construct according to any of claims 26 to 29, or a nucleic acid according to claim 16 for the preparation of a medicament for the treatment, prevention and/or alleviation of disorders or conditions relating to allergic reactions.

33. A polypeptide construct or nucleic acid according to claim 31 or a use of a polypeptide construct or nucleic acid according to claim 32 wherein said disorders are any of hay fever, asthma, atopic dermatitis, allergic skin reactions, allergic eye reactions and food allergies.

34. A polypeptide construct or nucleic acid according to claims 31 and 33 or a use of a polypeptide construct according to claim 32 and 33 wherein said polypeptide construct is administered intravenously, orally, sublingually, topically, nasally, vaginally, rectally or by inhalation.

35. A polypeptide construct according to any of claims 1 to 6 directed against a single target wherein said target is IFN-gamma .

36. A polypeptide construct according to claim 35 wherein said anti-target single domain antibodies are anti-IFN-gamma VHHs.

37. A polypeptide construct according to claim 35 wherein said anti-target single domain antibodies and said anti-serum protein antibodies are both VHHs .

38. A polypeptide construct according to any of claims 35 to 37, wherein said polypeptide construct is a homologous sequence of said polypeptide construct, a functional portion thereof, of an homologous sequence of a functional portion thereof.

39. A nucleic acid encoding a polypeptide construct according to any of claims 35 to 38.

40. A polypeptide construct according to any of claims 35 to 38, or a nucleic acid according to claim 39 for use in the treatment, prevention and/or alleviation of disorders or conditions wherein the immune system is over-active.

41. Use of a polypeptide construct according to any of claims 35 to 38, or a nucleic acid according to claim 39 for the preparation of a medicament for the treatment, prevention and/or alleviation of disorders or conditions wherein the immune system is over-active.

42. A polypeptide construct or nucleic acid according to claim 40 or a use of a polypeptide construct or nucleic acid according to claim 41 wherein said disorders are any of Crohn's disease, autoimmune disorders and organ plant rejection in addition inflammatory disorders such as rheumatoid arthritis, Crohn's disease, ulcerative colitis and multiple sclerosis.

43. A polypeptide construct or nucleic acid according to claims 40 and 42 or a use of a polypeptide construct according to claim 41 and 42 wherein said polypeptide construct is administered intravenously, orally, sublingually, topically, nasally, vaginally, rectally or by inhalation.

44. A composition comprising a polypeptide construct according to any of claims 7 to 10, 12, 14 and 15, or a nucleic acid encoding said polypeptide construct and a pharmaceutically acceptable vehicle.

45. A composition comprising a polypeptide construct according to any of claims 16 to 20, 22, 24 and 25, or a nucleic acid encoding said polypeptide construct and a pharmaceutically acceptable vehicle.

46. A composition comprising a polypeptide construct according to any of claims 26 to 29, 31, 33 and 34, or a nucleic acid encoding said polypeptide construct and a pharmaceutically acceptable vehicle.

47. A polypeptide construct according to any of claims 1 to 6 directed against a single target wherein said target is involved in a disease process.

48. A polypeptide construct according to claim 47, wherein said polypeptide construct is a homologous sequence of said polypeptide construct, a functional portion thereof, of an homologous sequence of a functional portion thereof.

49. A nucleic acid encoding a polypeptide construct according to claims 47 and 48.

50. A polypeptide construct according to claims 47 and 48, or a nucleic acid according to claim 49 for use in the treatment, prevention and/or alleviation of disorders or conditions in which the target is involved.

51. Use of a polypeptide construct according to any of claims 47 and 48, or a nucleic acid according to claim 49 for the preparation of a medicament for the treatment, prevention and/or alleviation of disorders or conditions in which the target is involved.

52. A polypeptide construct according to claims 48 and 50, or a nucleic acid according to claim 49 for use in treating, preventing and/or alleviating the symptoms of a disease requiring a therapeutic or diagnostic compound which is not rapidly cleared from the circulation.

53. Use of a polypeptide construct according to any of claims 48 and 50, or a nucleic acid according to claim 49 for the preparation of a medicament for treating, preventing and/or

alleviating the symptoms of a disease requiring a therapeutic or diagnostic compound which is not rapidly cleared from the circulation.

54. A polypeptide construct according to any of claims 48 and 50, or a nucleic acid according to claim 49 for use in treating, preventing and/or alleviating the symptoms of a disease requiring a therapeutic or diagnostic compound which remains active in the circulation for extended periods of time.

55. Use of a polypeptide construct according to any of claims 48 and 50, or a nucleic acid according to claim 49 for the preparation of a medicament for treating; preventing and/or alleviating the symptoms of a disease requiring a therapeutic or diagnostic compound which is remains active in the circulation for extended periods of time.

56. A polypeptide construct or nucleic acid according to any of claims 50, 52, 54, or use of a polypeptide construct or nucleic acid according to any of claims 51, 52, 53, 55, wherein said polypeptide construct is administered intravenously, orally, sublingually, topically, nasally, vaginally, rectally or by inhalation.

57. A composition comprising a polypeptide construct according to any of claims 47, 48, 50, 52, 54 and 56, or a nucleic acid according to any of claims 49, 50, 52, 54 and 56 and a pharmaceutically acceptable vehicle.

58. A method of producing a polypeptide according to any of claims 1 to 10, 16 to 20 , 26 to 29, 47 and 48 comprising

(a) culturing host cells comprising nucleic acid capable of encoding a polypeptide according to any of claims 1 to 10, 16 to 20 , 26 to 29, 47 and 48, under conditions allowing the expression of the polypeptide, and,

(b) recovering the produced polypeptide from the culture.

59. A method according to claim 58, wherein said host cells are bacterial or yeast.

60. A method for prolonging the half-life of a single domain antibody in the blood steam of a subject, said antibody directed against a therapeutic and/or diagnostic target by joining thereto one or more single domain antibodies each directed against a serum protein.

61. A method according to claim 60 wherein said anti-target single domain antibodies do not share the same sequence.

62. A method according to claim 60 wherein said anti-serum protein single domain antibodies do not share the same sequence.

63. A method according to claim 60 wherein said single domain antibodies are *Camelidae* VHHs antibodies.

64. A method according to any of claims 60 to 63 wherein said serum protein is any of serum albumin, serum immunoglobulins, thyroxine-binding protein, transferring, or fibrinogen or a fragment thereof.

65. A method according to any of claims 60 to 64 wherein said serum protein comprises a sequence corresponding to any of SEQ ID NOs: 1 to 3, a homologous sequence, a functional portion thereof, or a homologous sequence of a functional portion thereof.

66. A composition comprising a polypeptide according to any of claims 1 to 6 or a nucleic acid capable of encoding said polypeptide and a pharmaceutically acceptable vehicle.

ABSTRACT

The present invention relates to heterospecific polypeptide constructs comprising one or more single domain antibodies each directed against a therapeutic and/or diagnostic target and one or more single domain antibodies each directed against a serum protein, said construct having a prolonged lifetime in biological circulatory systems. The invention further relates to methods for stabilising VHHs in biological circulatory systems.

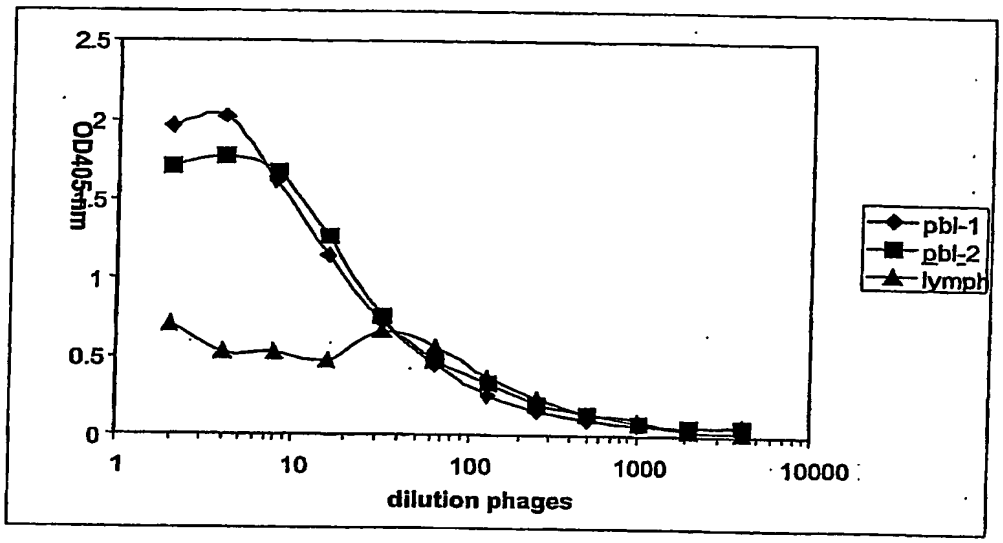

2.5
2
1.5
1
0.5
0
OD405nm
1
10
100
1000
10000
dilution phages
pbl-1
pbl-2
lymph

Fig. 1

SEQ ID NO	NAME	SEQUENCE
		Anti-mouse serum albumin
1	MSA 21	QVQLQESGGGLVQPGGSLRLSCEASGFTFSRFGMTWVRQAPGKGVEW VSGISSLGDSTLYADSVKGRFTISRDNAKNTLYLQMNSLKPEDTAVY YCTIGGSLNPGGQGTQVTVSS
2	MSA 24	QVQLQESGGGLVQPGNSLRLSCAASGFTFRNFGMSWVRQAPGKEPEW VSSISGSGSNTIYADSVKDRFTISRDNAKSTLYLQMNSLKPEDTAVY YCTIGGSLSRSSQGTQVTVSS
3	MSA 210	QVQLQESGGGLVQPGGSLRLTCTASGFTFSSFGMSWVRQAPGKGLEW VSAISSDSGTKNYADSVKGRFTISRDNAKKMLFLQMNSLRPEDTAVY YCVIGRGSPSSQGTQVTVSS
		Anti-mouse serum albumin/anti-TNF-alpha
4	MSA 21/TNF3E	QVQLQESGGGLVQPGGSLRLSCEASGFTFSRFGMTWVRQAPGKGVEW VSGISSLGDSTLYADSVKGRFTISRDNAKNTLYLQMNSLKPEDTAVY YCTIGGSLNPGGQGTQVTVSSEPKTPKPQPAAAQVQLQESGGGLVQP GGSLRLSCAASGRTFSDHSGYTYTIGWFRQAPGKEREFVARIYWSSG NTYYADSVKGRFAISRDIAKNTVDLTMNNLEPEDTAVYYCAARDGIP TSRSVESYNYWGQGTQVTVSS
5	MSA 24/TNF3E	QVQLQESGGGLVQPGNSLRLSCAASGFTFRNFGMSWVRQAPGKEPEW VSSISGSGSNTIYADSVKDRFTISRDNAKSTLYLQMNSLKPEDTAVY YCTIGGSLSRSSQGTQVTVSSEPKTPKPQPAAAQVQLQESGGGLVQP GGSLRLSCAASGRTFSDHSGYTYTIGWFRQAPGKEREFVARIYWSSG NTYYADSVKGRFAISRDIAKNTVDLTMNNLEPEDTAVYYCAARDGIP TSRSVESYNYWGQGTQVTVSS
6	MSA 210/TNF3E	QVQLQESGGGLVQPGGSLRLTCTASGFTFSSFGMSWVRQAPGKGLEW VSAISSDSGTKNYADSVKGRFTISRDNAKKMLFLQMNSLRPEDTAVY YCVIGRGSPSSQGTQVTVSSEPKTPKPQPAAAQVQLQESGGGLVQPG GSLRLSCAASGRTFSDHSGYTYTIGWFRQAPGKEREFVARIYWSSGN TYYADSVKGRFAISRDIAKNTVDLTMNNLEPEDTAVYYCAARDGIPT SRSVESYNYWGQGTQVTVSS

Fig. 2

		Anti-IgE
7	EV 2C3	QVQLQDSGGGLVQPGGSLRLSCRASGRIFRINAMGWYRQAPGKQREL VATITSTGSTNFADSVKGRFTIYRDGAKRTVDLRLNSLKPEDTAVYF CNADVREYDLGPWRQYWGQGTQVTVSS
8	EV 2C1	QVQLQESGGGLVQPGDSLRLSCVVSGRTLSYSSLAWFRQAPGKERDF VAALSLTTYYADSVKGRFTISRDNAKNTVYLQMNSLKPDDTADYFCA TARTRTDYAPLLSAASTYDAWGQGTQVTVSL
9	EV 2H3	QVQLQESGGGLVQAGGSLRLSCAASGRSSRYYAMGWFRQGPGKEREF VAAVNWNGDSTYYADSVKGRFTISRGNAENTAYLQMNSLVPEDTAVY YCAMRMNAGLGYSAASYQYWGQGTQVTVSL
10	EV 2D12	QVQLQESGGGLVQAGDSLRLSCAASGLTFLEHVMAWFRQTPGKEREF VGAIDWSGRRITYTDSVKGRFTISRDNAKNTVYLQMNTLKPEDTAVY YCAADRTYSYSSTGYYYWGQGTQVTVSS
11	EV 2G4	QVQLQDSGGGLVQAGDSLRLSCAASGLTFLEHVMAWFRQTPGKEREF VGAIDWSGRRITYTDSVKGRFTISRDNAKNTVYLQMNTLKPEDTAVY YCAADRTYSYSSTGYYYWGQGTQVTVSS
12	EV 4C5	QVQLQESGGGLVQAGGSLRLSCAASGRTLSSYTMAWFRQAPGKEREF VASISSSGISTYYADSVKGRFTISRDIAKNTVYLQMNSLKPEDTAVY YCAAKYRYYSTLYTKSGEYDYWGQGTQVTVSS
13	EV 4A2	QVQLQDSGGGLVQAGGSLRLSCEASGRTISSYAMAWFRQAPGKEREF VASISSSGVSKHYADSVKGRFTISNDKVKNTVYLQMNSLKPEDTAVY FCAAKYRYYSSYYTKSGDYDYWGQGTQVTVSS
14	EV 2D4	QVQLQESGGGLVQAGGSLRLSCAASGLTFSTYAMGWFRQAPGKEREF VAAVSYSGSYYADSVKGRFTISRDNAKNTVYLQMASLKPEDTAVYYC AARNRGYSTYAGVYDYWGQGTQVTVSS
15	EV 2B6	QVQLQDSGGGLVQAGGSLRLSCAASGVTFSSYAMGWFRQAPGKEREF VASITWIGGGTYYADSVKGRFTISRDHAGNTVYLQMNTLKPDDTAVY YCALDRRSSTYYLMKGEYDYRGRGTQVTVSS
16	EV 2H11	QVQLQESGGGLVQAGGSLRLSCAASGVTFSSYAMGWFRQAPGKEREF VASITWTGTGTYYADSVKGRFTISRDHAGTTVYLQMNSLKPEDTAVY YCAVDRRSSTYYLMKGEYDYRGRGTQVTVSS

Fig. 2

		Anti-TNF-alpha
17	VHH#1	QVQLQESGGGLVQPGGSLRLSCATSGFDFSVSWMYWVRQAPGKGLEW VSEINTNGLITKYVDSVKGRFTISRDNAKNTLYLQMDSLIPEDTALY YCARSPSGSFRGQGTQVTVSS
18	VHH#9	QVQLQESGGGLVQPGGSLRLSCAASGSIFRVNAMGWYRQVPGNQREF VAIITSGDNLNYADAVKGRFTISTDNVKKTVYLQMNVLKPEDTAVYY CNAILQTSRWSIPSNYWGQGTQVTVSS
19	VHH#13	QVQLQESGGGLVQPGGSLRLSCATSGFTFSDYWMYWVRQAPGKGLEW VSTVNTNGLITRYADSVKGRFTISRDNAKYTLYLQMNSLKSEDTAVY YCTKVVPPYSDDSRTNADWGQGTQVTVSS
20	VHH#2	QVQLQESGGGLVQPGGSLRLSCAASGRTFSDHSGYTYTIGWFRQAPGK EREFVARIYWSSGNTYYADSVKGRFAISRDIAKNTVDLTMNNLEPEDT AVYYCAARDGIPTSRSVESYNYWGQGTQVTVSS
21	VHH#3	QVQLQDSGGGLVQAGGSLRLSCAVSGRTFSAHSVYTMGWFRQAPGKER EFVARIYWSSANTYYADSVKGRFTISRDNAKNTVDLLMNSLKPEDTAV YYCAARDGIPTSRTVGSYNYWGQGTQVTVSS
22	VHH#4	QVQLQESGGGLVQPGGSLRLSCAASGSIFRVNAMGWYRQVPGNQREFV AIITSSDTNDTTNYADAVKGRFTISTDNVKKTVYLQMNVLKPEDTAVY YCNAVLQTSRWSIPSNYWGQGTQVTVSS
23	VHH#5	QVQLQDSGGGLVQAGGSLRLSCTTSGRTISVYAMGWFRQAPGKEREFV ASISGSGAITPYADSVKGRFTISRDNAKNTVYLQMNSLNPEDTAVYYC AASRYARYRDVHAYDYWGQGTQVTVSS
24	VHH#6	QVQLQDSGGGLVQAGGSLRLSCAASTRTFSRYVVGWFRQAPGKEREFV ATISWNGEHTYYADSVKGRYTISRDNAKNTVYLQMGSLKPEDTAVYYC AARSFWGYNVEQRDFGSWGQGTPVTVSS
25	VHH#7	QVQLQESGGGLVQPGGSLRLSCAASGSIFRVNAMGWYRQVPGNQREFV AIITNDTTNYADAVKGRFTISTDNVKKTVYLQMNVLKPEDTAVYYCNT VLQTSRWNIPTNYWGQGTQVTVSS
26	VHH#8	QVQLQESGGGLVQPGGSLRLSCAASGSIFRVNAMGWYRQVPGNQREFV AIISGDTTNYADAVKGRFTISTDNVKKTVYLQMNVLESEDTAVYYCNA VLQTSRWSIPSNYWGQGTQVTVSS
27	VHH#10	QVQLQDSGGGLVQPGGSLRLACVASGSIFSIDVMGWYRQAPGQQRELV ATITNSWTTNYADSVKGRFTISRDNAKNVVYLQMNSLKLEDTAVYYCN ARRWYQPEAWGQGTQVTVSS
28	VHH#11	QVQLQDSGGGLVQPGGSLRLSCAASGFTFSTHWMYWVRQAPGKGLEWV STINTNGLITDYIHSVKGRFTISRDNAKNTLYLQMNSLKSEDTAVYYC ALNQAGLSRGQGTQVTVSS
29	VHH#12	QVQLQESGGGLVQAGGSLRLSCAASRRTFSGYAMGWFRQAPGKEREFV AVVSGTGTIAYYADSVKGRFTISRDNAENTVYLQMNSLKPEDTGLYYC AVGPSSSRWYYRGASLVDYWGKGTLVTVSS

Fig. 2

		Anti-vWF
30	C37	QVQLQESGGGLVQPGGSLRLSCAASGFNFNWYPMSWVRQAPGKGLEW VSTISTYGEPRYADSVKGRFTISRDNANNTLYLQMNSLRPEDTAVYY CARGAGTSSYLPQRGNWDQGTQVTISS
31	T76	QVQLQESGGGLVQPGESLRLSCAASGSIFSINTMGWYGQAPGKQREL VASITFGGVTNYADSVKGRFTISRDNTNDTVYLQMNSLKPEDTAVYI CNAVTWGGLTNYWGQGTQVTVSS
32	Z29	QVQLQESGGGSVQAGDSLTLSCAASGRTFSMHAMGWFRQAPGKEREF VAAISPSAFTTEYADSLKGRFTVSRDNAKKLVWLQMNGLKPEDTAAY YCAARRGAFTATTAPLYDYWGQGTQVTVSS
33	A50	QVQLQESGGGLVQAGGSLRLSCAASGRTFSSYRMGWFRQAPGKEREF VAAISRRGDNVYYADSVKGRFAISRDNAESTLYLQMNSLKPEDTAVY YCAAHVTVSAITLSTSTYDYWGQGTQVTVSS
34	A38	QVQLQDSGGGSVQAGGSLRLSCAASGRTVSSYNMGWFRRVPGKERDF VAAISWSGVATYYFDSVKGRFTISRDNAKNTVYLEMNSLKPEDTAVY YCAAASRYRHRLNSGSEYDYWGQGTQVTVSS
35	I53	QVQLQDSGGGLVQAGGSLRLSCAASGRTKDMAWFRQPPGKEREFVAV IYSSDGSTLVAASVKGRFTISRDNAKNTVYLQMTSLKPADTAVYYCA TSRGYSGTYYSTSRYDYWTGGTQVTVSS
36	M53	QVQLQDSGGGLVQAGESLRLSCGTSGRTFGRRAMAWFRQAPGKERQF VAWIARYDGSTLYADSVKGRFTISRDDNKNTMYLHMNNLTPEDTAVY YCAAGPRGLYYESRYEYWGQGTLVTVSS
37	2L-34	QVQLQDSGGGLVQAGGSLRLSCAASVRIFTSYAMGWFRQAPGKEREF VAAINRSGKSTYYSDSVEGRFTISRDNAKNTVSLQMDSLKLEDTAVY YCAADYSGSYTSLWSRPERLDWGQGTQVTVFS
38	4L-16	QVQLVESGGGLVQAGGSLRLSCAASGRTFSSYAMGWFRQAPGKEREF VAAISWSGGSTYYADSVKGRFTISRDNAKNTVYLQMNSLKPEDTAVY YCVADTGGISWIRTQGYNYWGQGTQVTVSS
39	AM-4-15-3	QVQLQDSGGGLVQPGGSLRLACAASGSIFSINSMGWYRQAPGKQREL VAHALADGSASYRDSVKGRFTISRDNAKNTVYLQMNSLKPEDTAVYY CNTVPSSVTKGYWGQGTQVTVSS
		Anti-mouse serum albumin/anti-vWF
40	MSA 21/ AM-4-15-3	QVQLQESGGGLVQPGGSLRLSCEASGFTFSRFGMTWVRQAPGKGVEW VSGISSLGDSTLYADSVKGRFTISRDNAKNTLYLQMNSLKPEDTAVY YCTIGGSLNPGGQGTQVTVSSEPKTPKPQPAAAQVQLQDSGGGLVQP GGSLRLACAASGSIFSINSMGWYRQAPGKQRELVAHALADGSASYRD SVKGRFTISRDNAKNTVYLQMNSLKPEDTAVYYCNTVPSSVTKGYWG QGTQVTVSS
41	MSA 24/ AM-4-15-3	QVQLQESGGGLVQPGNSLRLSCAASGFTFRNFGMSWVRQAPGKEPEW

Fig. 2

		VSSISGSGSNTIYADSVKDRFTISRDNAKSTLYLQMNSLKPEDTAVY YCTIGGSLSRSSQGTQVTVSSEPKTPKPQPAAAQVQLQDSGGGLVQP GGSLRLACAASGSIFSINSMGWYRQAPGKQRELVAHALADGSASYRD SVKGRFTISRDNAKNTVYLQMNSLKPEDTAVYYCNTVPSSVTKGYWG QGTQVTVSS
42	MSA 210/ AM-4-15-3	QVQLQESGGGLVQPGGSLRLTCTASGFTFSSFGMSWVRQAPGKGLEW VSAISSDSGTKNYADSVKGRFTISRDNAKKMLFLQMNSLRPEDTAVY YCVIGRGSPSSQGTQVTVSSEPKTPKPQPAAAQVQLQDSGGGLVQPG GSLRLACAASGSIFSINSMGWYRQAPGKQRELVAHALADGSASYRDS VKGRFTISRDNAKNTVYLQMNSLKPEDTAVYYCNTVPSSVTKGYWGQ GTQVTVSS

		Anti-mouse serum albumin/anti-IgE
43	MSA 21/ EV 2H11	QVQLQESGGGLVQPGGSLRLSCEASGFTFSRFGMTWVRQAPGKGVEW VSGISSLGDSTLYADSVKGRFTISRDNAKNTLYLQMNSLKPEDTAVY YCTIGGSLNPGGQGTQVTVSSEPKTPKPQPAAAQVQLQESGGGLVQA GGSLRLSCAASGVTFSSYAMGWFRQAPGKEREFVASITWTGTGTYYA DSVKGRFTISRDHAGTTVYLQMNSLKPEDTAVYYCAVDRRSSTYYLM KGEYDYRGRGTQVTVSS
44	MSA 24/ EV 2H11	QVQLQESGGGLVQPGNSLRLSCAASGFTFRNFGMSWVRQAPGKEPEW VSSISGSGSNTIYADSVKDRFTISRDNAKSTLYLQMNSLKPEDTAVY YCTIGGSLSRSSQGTQVTVSSEPKTPKPQPAAAQVQLQESGGGLVQA GGSLRLSCAASGVTFSSYAMGWFRQAPGKEREFVASITWTGTGTYYA DSVKGRFTISRDHAGTTVYLQMNSLKPEDTAVYYCAVDRRSSTYYLM KGEYDYRGRGTQVTVSS
45	MSA 210/ EV 2H11	QVQLQESGGGLVQPGGSLRLTCTASGFTFSSFGMSWVRQAPGKGLEW VSAISSDSGTKNYADSVKGRFTISRDNAKKMLFLQMNSLRPEDTAVY YCVIGRGSPSSQGTQVTVSSEPKTPKPQPAAAQVQLQESGGGLVQAG GSLRLSCAASGVTFSSYAMGWFRQAPGKEREFVASITWTGTGTYYAD SVKGRFTISRDHAGTTVYLQMNSLKPEDTAVYYCAVDRRSSTYYLMK GEYDYRGRGTQVTVSS

		Anti-IFN-gamma
46	MP2B5BR	QVQLVESGGRLVQAGGSLRLSCIASGRTISDYAAGWFRQAPGKEREFLASVT WGFGSTSYADSVKGRFTISRDKAKDTVYLQMNTLEPDDTSVYYCASSPRYCA GYRCYVTASEFDSWGQGTQVTVSS
47	MP2C10BR	QVKLEESGGGLVQAGGSLRLSCAASGLTYTVGWFRQAPGKEREFVAAISWSG GSALYADSVKGRFTISRDNAKNTVYLQMGSLEPEDTAYYSCAAPGTRYYGSN QVNYNYWGQGTQVTVSS
48	MP2D5BR	AVQLVESGGGLVQAGDSLRLSCAASGRSFSSYGMGWFRQAPGKEHEFVAGIW RSGVSLYYADSVKGRFTISRDDAKMTVSLEMNSLKPEDTAVYYCAAEATFPT WNRGRFADYDYRGQGTQVTVSS

Fig. 2

49	MP2F1BR	AVQLVESGGGLVQTGDSLRLSCVASGGTFSRYAMGWFRQAPGKEREFVARIG YSGRSISYATSVEGRFAISRDNAKNTVYLQMNSLKPEDTAVYYCASLVSGTL YQADYWGQGTQVTVSS
50	MP2F10SR	QVQLVESGGGLVQAGGSLRLSCAASGRTISSFRMGWFRRAPGEEREFVAFVR SNGTSTYYADSVEGRFTITRDNAKNTVYLRMDSLKPEDTAVYYCAAATRDYG GSFDYWGQGTQVTVSS
51	MP2F6SR	QVKLEESGGGLVQAGGSLRLSCAASGRTFNNYNMGWFRQAPGKEREFVAAIS WNGGSTYYDDSVKGRFTISRDNANNLVYLQMNSLNFEDTAVYYCACAANPYG IPQYRENRYDFWGQGTQVTVSS
52	MP3D2BR	QVQLQESGGGLVQPGESLRLSCAASRGIFRFNAGGWYRQAPGKQRELVAFIG VDNTTRYIDSVKGRFTISRDNAKTTVYLQMNSLQPEDTAVYYCNKVPYIDWG QGTQVTVSS
53	MP3E5BR	QVQLQESGGGLVQAGDSLRLSCAASGRSFSSYGMGWFRQAPGKEHEFVAGIW RSGVSLYYADSVKGRFTISRDDAKMTVSLQMNGLKPEDTAVYYCAAEATFPT WNRGSFADYDYRGQGTQVTVSS
54	MP3D1BR	QVQLQESGGGLVQAGGSLRLSCAASGRTFDNYNMGWFRQAPGKEREFVAAIS WNGGSTYYDDSVKGRFTISRDNFQKLVYLQMNSLKLEDTAVYYCACAANPYG IPQYRENRYDFWGQGTQVTVSS
55	MP3B1BR	QVQLQDSGGGLVQAGGSLRLSCAASGRTFSTYNMGWFRQAPGKEREFVAGIS WNGGSIYYTSSVEGRFTISRDNAENTVYLQMNSLKPEDTGVYYCASKGRPYG VPSPRQGDYDYWGQGTQVTVSS
56	MP3H1SR	QVQLQESGGGLVQAGGSLRLSCAAAGISGSVFSRTPMGWYRQAPGKQRELVA GILTSGATSYAESVKGRFTISRDNAKNTVYLQMNSLSPEDTAEYYCNTYPTW VLSWGQGTQVTVSS
57	MP3A12SRA	QVQLQDSGGGLVQAGGSLRLSCAAAGISGSVFSRTPMGWYRQAPGKQRELVA GILTSGATSYAESVKGRFTISRDNAKNTVYLQMNSLSPEDTAEYYCNTYPTW VLSWGQGTQVTVSS
58	MP3A7SRA	QVQLQDSGGGLVQAGGSLRLSCAASGRTFSSFRMGWFRRAPGEEREFVAFVR SNGTSTYYADSVEGRFTITRDNAKNTVYLRMDSLKPEDTAVYYCAAATRDYG GSFDYWGQGTQVIVSS
59	MP3C7SRA	QVQLQESGGGLVQAGDSLRLSCAASGRSFSSYGMGWFRQAPGKEHEFVAGIW RSGVSLYYADSVKGRFTISRDDAKMTVSLQMNSLKPEDTAVYYCAAEATFPT WNRGRFADYDYSGQGTQVTVSS
60	MP3D2SRA	QVQLQDSGGGTVQAGGSLRLSCAASGRTFSDYAVGWFRQAPGKEREFVARIL WTGASRSYANSVDGRFTVSTDNAKNTVYLQMNSLKPEDTAIYYCAALPSNII TTDYLRVYYWGQGTQVTVSS
61	MP3F1SRA	QVQLQESGGGLVQAGGSLTLSCVASGRTISDYAVGWFRQAPGKEREFVASIS WGGGFTAFADSMKGRFTISRDNAKNTVYLQTHTLEPDDTSVYYCASSRRYCT GYRCYATASEFDSWGQGTQVTVSS
62	MP3H6SRA	QVQLQESGGGLVQAGGSLRLSCAASGRTFSTYNMGWFRQAPGKEREFVAGIS WNGGSIYYTSSVEGRFTISRDNAENTVYLQMNSLKPEDTGVYYCASKGRPYG VPSPRQGDYDYWGQGTQVTVSS
		Anti-mouse serum albumin/anti-IFN-gamma
63	MSA 21/ MP2F6SR	QVQLQESGGGLVQPGGSLRLSCEASGFTFSRFGMTWVRQAPGKGVEW VSGISSLGDSTLYADSVKGRFTISRDNAKNTLYLQMNSLKPEDTAVY

Fig. 2

		YCTIGGSLNPGGQGTQVTVSSEPKTPKPQPAAAQVKLEESGGGLVQA GGSLRLSCAASGRTFNNYNMGWFRQAPGKEREFVAAISWNGGSTYYD DSVKGRFTISRDNANNLVYLQMNSLNFEDTAVYYCACAANPYGIPQY RENRYDFWGQGTQVTVSS
64	MSA 24/ MP2F1BR	QVQLQESGGGLVQPGNSLRLSCAASGFTFRNFGMSWVRQAPGKEPEW VSSISGSGSNTIYADSVKDRFTISRDNAKSTLYLQMNSLKPEDTAVY YCTIGGSLSRSSQGTQVTVSSEPKTPKPQPAAAAVQLVESGGGLVQT GDSLRLSCVASGGTFSRYAMGWFRQAPGKEREFVARIGYSGRSISYA TSVEGRFAISRDNAKNTVYLQMNSLKPEDTAVYYCASLVSGTLYQAD YWGQGTQVTVSS
65	MSA 210/ MP3H6SRA	QVQLQESGGGLVQPGGSLRLTCTASGFTFSSFGMSWVRQAPGKGLEW VSAISSDSGTKNYADSVKGRFTISRDNAKKMLFLQMNSLRPEDTAVY YCVIGRGSPSSQGTQVTVSSEPKTPKPQPAAAQVQLQESGGGLVQAG GSLRLSCAASGRTFSIYNMGWFRQAPGKEREFVAGISWNGGSIYYTS SVEGRFTISRDNAENTVYLQMNSLKPEDTGVYYCASKGRPYGVPSPR QGDYDYWGQGT QVTVSS

Fig. 2

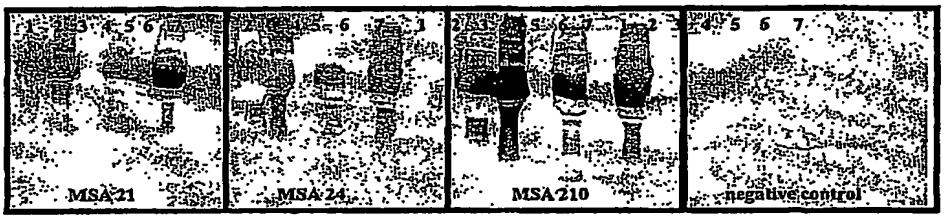
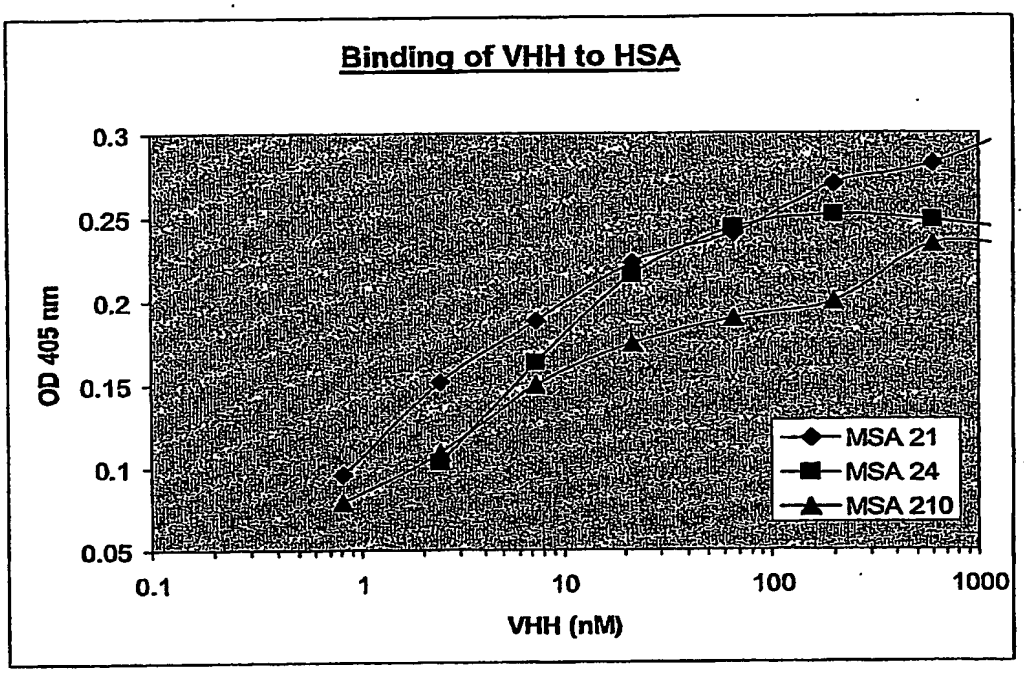


Fig. 3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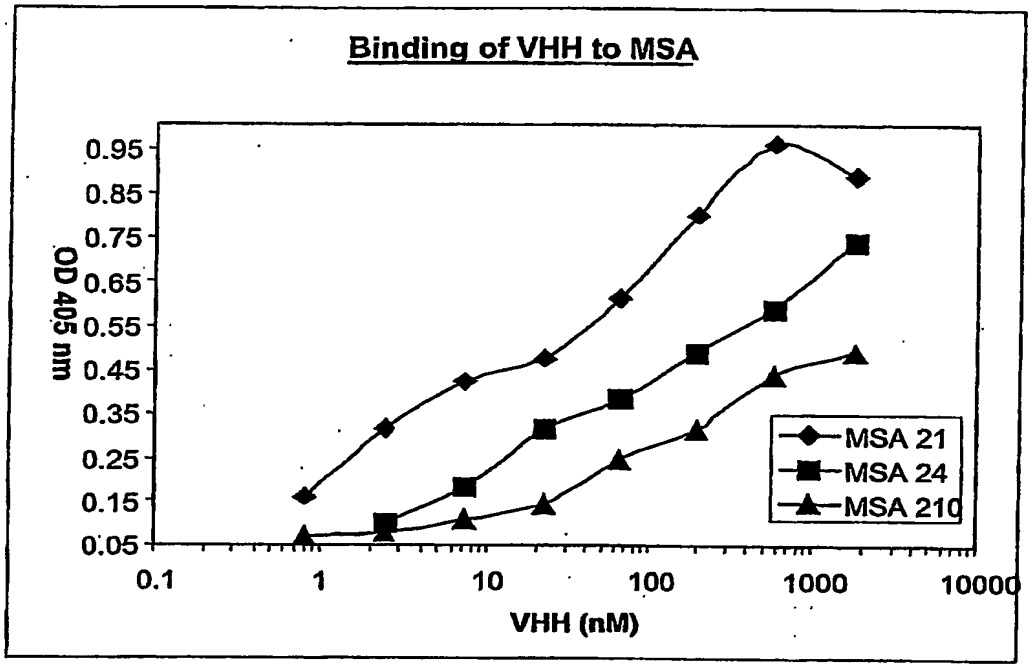
0.25
0.2
0.15
0.1
0.05
0.1
1
10
100
1000
VHH (nM)
MSA 21
MSA 24
MSA 210

Fig. 4


Binding of VHH to MSA
OD 405 nm
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0.85
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0.45
0.35
0.25
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0.05
0.1
1
10
100
1000
10000
VHH (nM)
MSA 21
MSA 24
MSA 210

Fig. 5

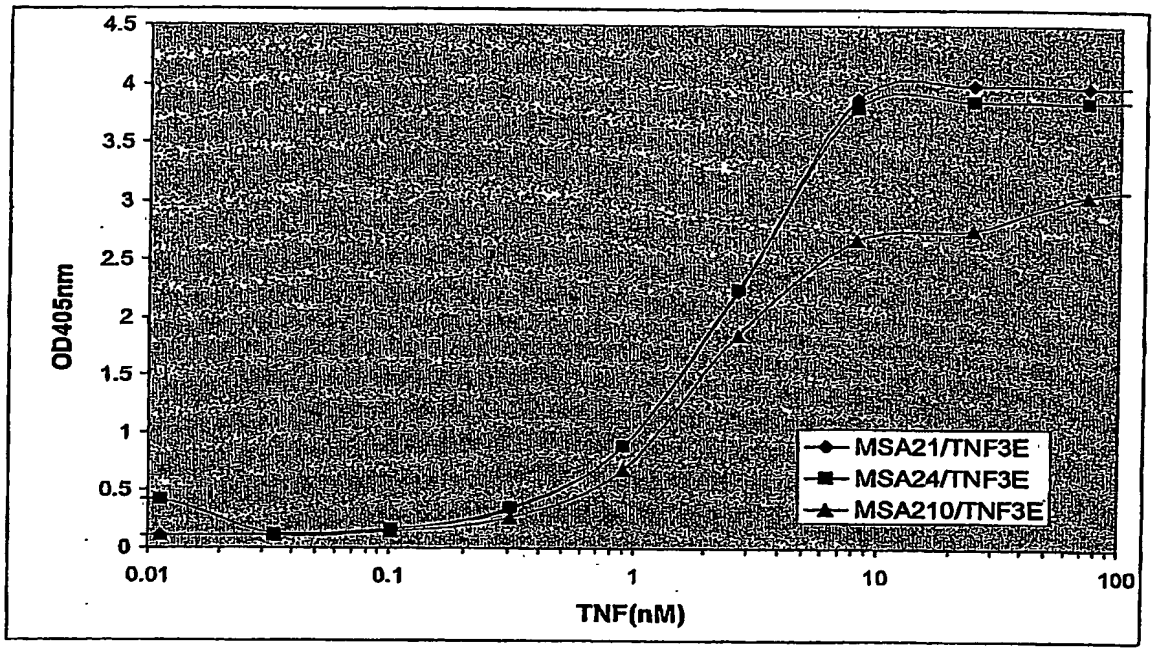
4.5
4
3.5
3
2.5
2
1.5
1
0.5
0
OD405nm
0.01
0.1
1
10
100
TNF(nM)
MSA21/TNF3E
MSA24/TNF3E
MSA210/TNF3E

Fig. 6

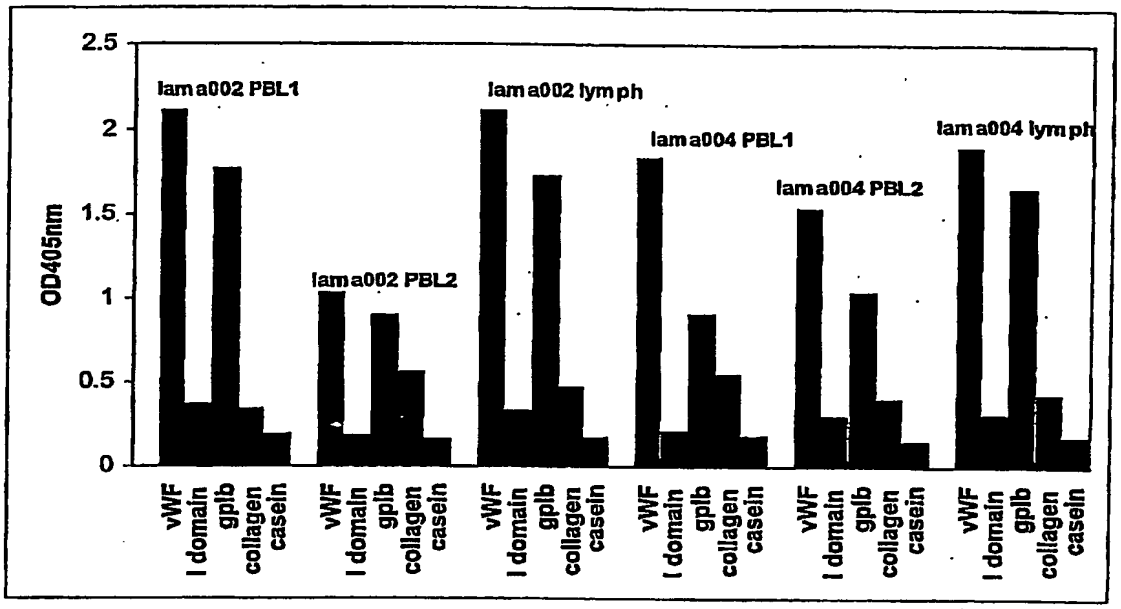
2.5
2
1.5
1
0.5
0
OD405nm
lama002 PBL1
lama002 PBL2
lama002 lymph
lama004 PBL1
lama004 PBL2
lama004 lymph
vWF
I domain
gpIb
collagen
casein

Fig. 7

A1 domain

A3 domain

Fig. 8

Sequences A1 domain specific binders:

```
        --------framework 1---------CDR1-------framework 2-------CDR2-
A50     LQESGGGLVQAGGSLRLSCAASGRTFS SYRMG WFRQAPGKEREFVAAISRRGDN VYYAD
A38     LQDSGGGSVQAGGSLRLSCAASGRTVS SYNMG WFRRVPGKERDFVAAISWSGVA TYYFD
I53     LQDSGGGLVQAGGSLRLSCAASGRTKD MA    WFRQPPGKEREFVAVIYSSDGS TLVAA
M53     LQDSGGGLVQAGESLRLSCGTSGRTFG RRAMA WFRQAPGKERQFVAWIARYDGS TLYAD
Z29     LQESGGGSVQAGDSLTLSCAASGRTFS MHAMG WFRQAPGKEREFVAAISPSAFT TEYAD

        ------framework 3----------------------CDR3---------
A50     SVKGRFAISRDNAESTLYLQMNSLKPEDTAVYYCAA HVTVSAITLSTSTYDY
A38     SVKGRFTISRDNAKNTVYLEMNSLKPEDTAVYYCAA ASRYRHRLNSGSEYDY
I53     SVKGRFTISRDNAKNTVYLQMTSLKPADTAVYYCAT SRGYSGTYYSTSRYDY
M53     SVKGRFTISRDDNKNTMYLHMNNLTPEDTAVYYCAA   GPRGLYY ESRYEY
Z29     SLKGRFTVSRDNAKKLVWLQMNGLKPEDTAAYYCAA RRGAFTATTAP LYDY

        framework4-
A50     WGQGTQVTVSS
A38     WGQGTQVTVSS
I53     WTGGTQVTVSS
M53     WGQGTLVTVSS
Z29     WGQGTQVTVSS
```

Sequence of A3 domain specific binders:

```
        --------framework 1---------CDR1-------framework 2-------CDR2-
C37     LQESGGGLVQPGGSLRLSCAASGFNFN WYPMS WVRQAPGKGLEWVSTISTYGEPR  YAD
T76     LQESGGGLVQPGESLRLSCAASGSIFS INTMG WYGQAPGKQRELVASITFGGVTN  YAD

        ------framework 3----------------------CDR3---------
C37     SVKGRFTISRDNANNTLYLQMNSLRPEDTAVYYCAR GAGTSSYLPQRGN
T76     SVKGRFTISRDNTNDTVYLQMNSLKPEDTAVYICNA VTWGGLTNY

        framework4-
C37     WDQGTQVTISS
T76     WGQGTQVTVSS
```

Fig. 9

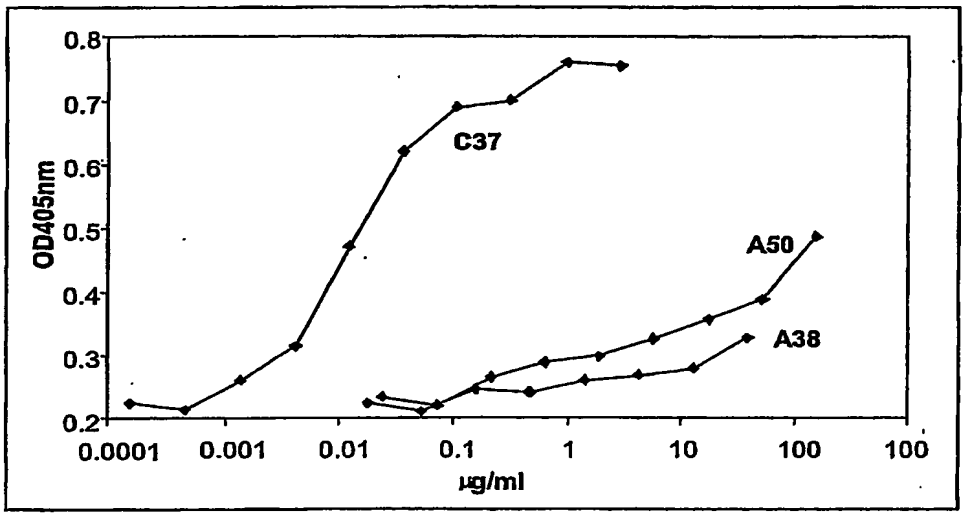
0.8
0.7
0.6
0.5
0.4
0.3
0.2
OD405nm
C37
A50
A38
0.0001
0.001
0.01
0.1
1
10
100
100
µg/ml

Fig. 10

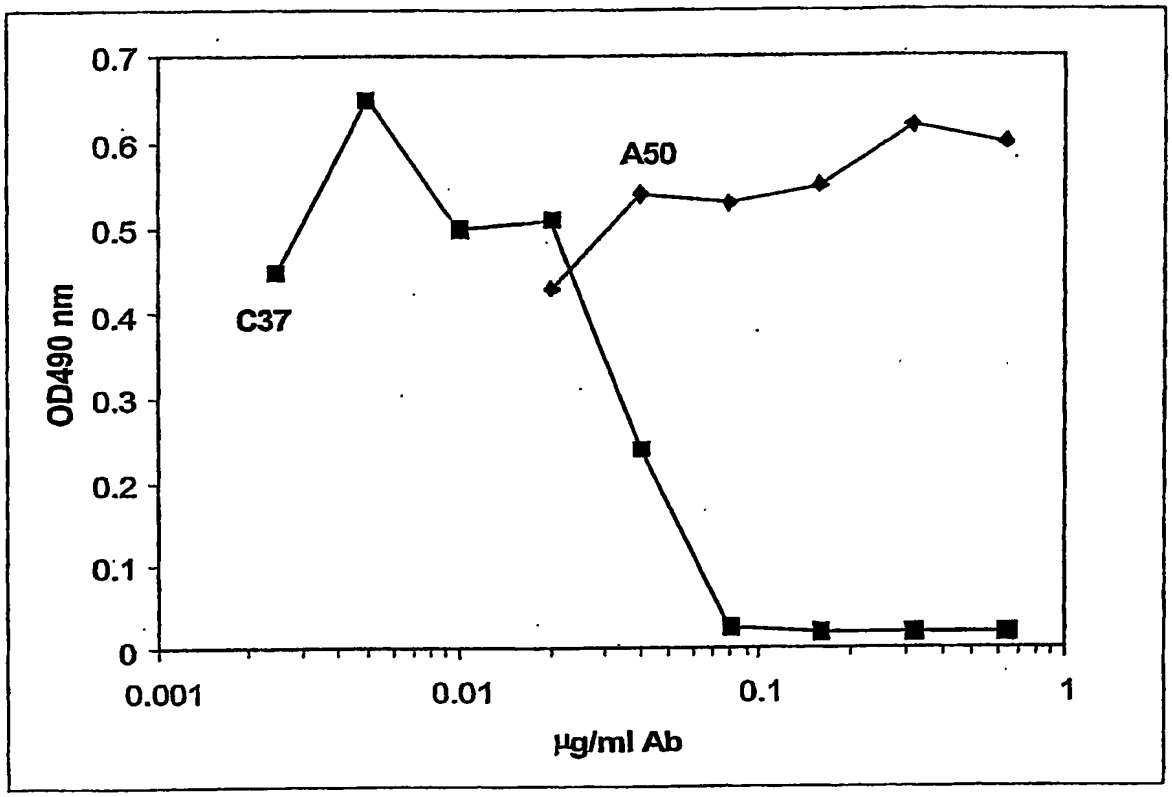

Fig. 11

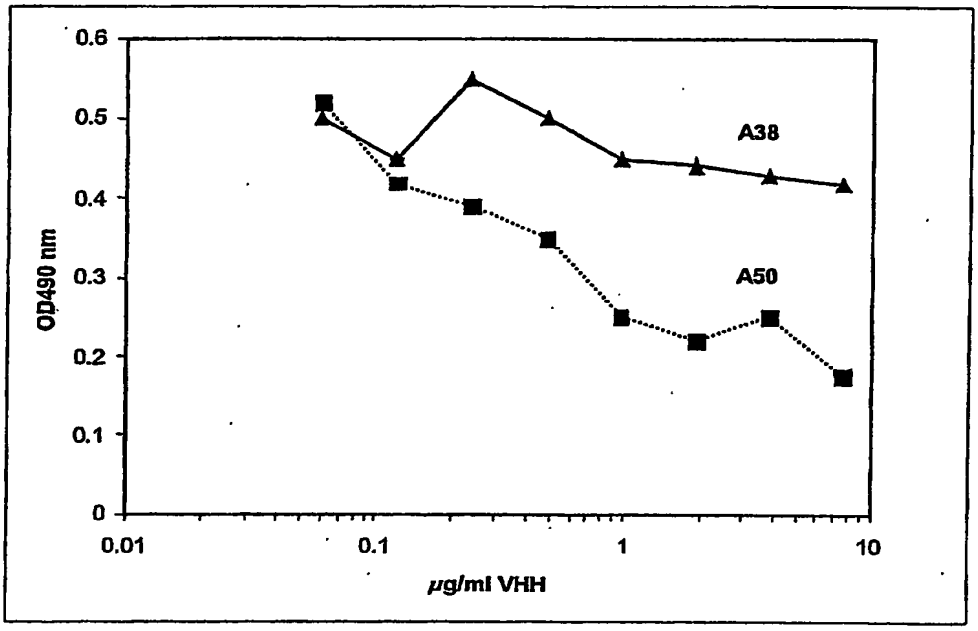

Fig. 12

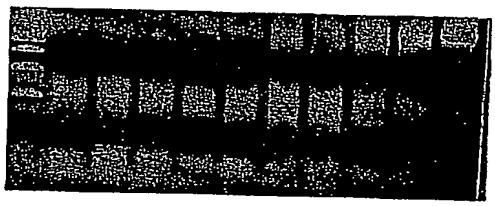

Fig. 13

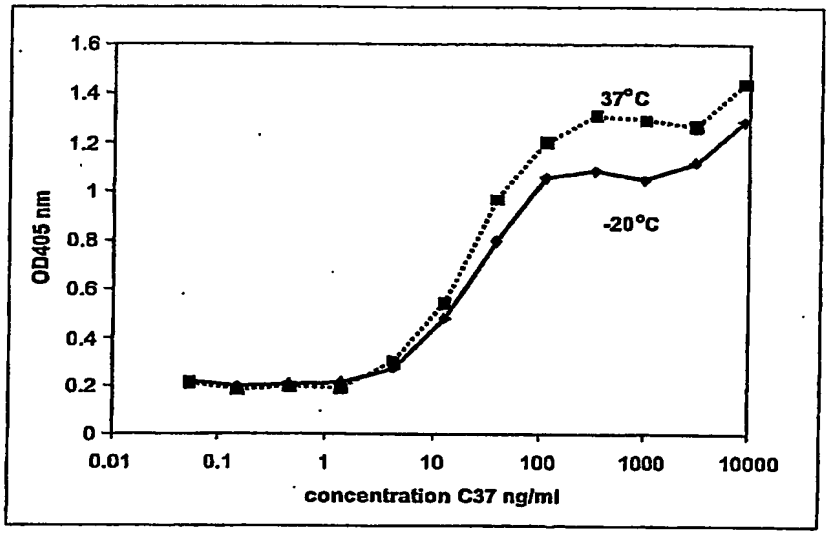
1.6
1.4
1.2
1
0.8
0.6
0.4
0.2
0
OD405 nm
37°C
-20°C
0.01
0.1
1
10
100
1000
10000
concentration C37 ng/ml

Fig. 14

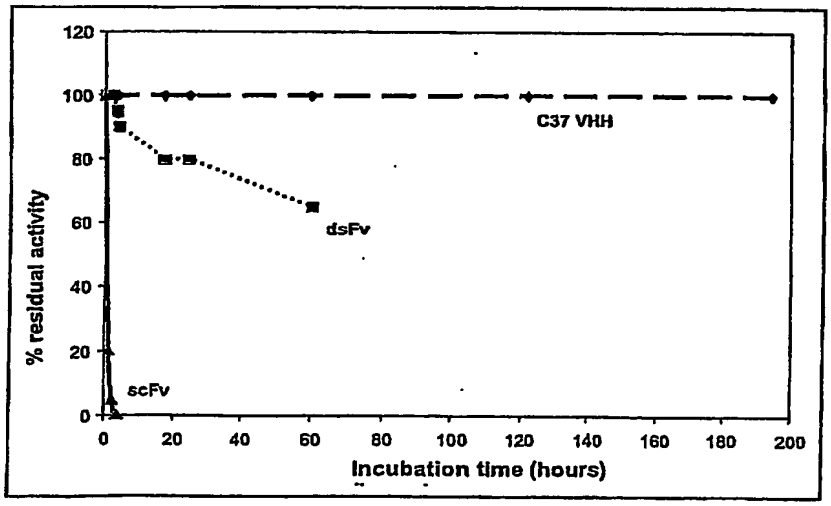
120
100
80
60
40
20
0
% residual activity
C37 VHH
dsFv
scFv
0
20
40
60
80
100
120
140
160
180
200
Incubation time (hours)

Fig. 15